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- (74) Agents: YAMAMOTO, Shusaku et al.; Crystal Tower,
15th Floor, 2-27, Shiromi 1-chome, Chuo-ku, Osaka-shi,
Osaka 5406015 (JP).
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ZW.
- (71) Applicant (*for all designated States except US*): NA-
TIONAL INSTITUTE OF ADVANCED INDUSTRIAL
SCIENCE AND TECHNOLOGY [JP/JP]; 1-3-1, Ka-
sumigaseki, Chiyoda-ku, Tokyo 1008921 (JP).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): MIYAKE,
Masato [JP/JP]; c/o Aist Kansai, Amagasaki Site,
11-46, Nakoji 3-chome, Amagasaki-shi, Hyogo 6610974
(JP). YOSHIKAWA, Tomohiro [JP/JP]; c/o Aist Kansai,
Amagasaki Site, 11-46, Nakoji 3-chome, Amagasaki-shi,
Hyogo 6610974 (JP). UCHIMURA, Eiichiro [JP/JP];
c/o Aist Kansai, Amagasaki Site, 11-46, Nakoji 3-chome,
Amagasaki-shi, Hyogo 6610974 (JP). MIYAKE, Jun
[JP/JP]; c/o Aist Kansai, Amagasaki Site, 11-46, Nakoji
3-chome, Amagasaki-shi, Hyogo 6610974 (JP).
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(54) Title: COMPOSITION AND METHOD FOR INCREASING EFFICIENCY OF INTRODUCTION OF TARGET
SUBSTANCE INTO CELL

(57) Abstract: The present invention provides a method capable of improving the efficiency of introducing a target substance (e.g., DNA, polypeptides, sugars, or complexes thereof), which is difficult to introduce (particularly, transfect) into a cell in any circumstances. Particularly, the present invention provides a composition for increasing the efficiency of introducing a target substance into a cell, comprising (a) an actin acting substance. The present invention also provides a device and method using such a composition.

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DESCRIPTION

COMPOSITION AND METHOD FOR INCREASING EFFICIENCY OF
INTRODUCTION OF TARGET SUBSTANCE INTO CELL

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TECHNICAL FIELD

The present invention relates to the field of cell biology. More particularly, the present invention relates to a compound, composition, device, method and system for increasing the efficiency of introducing a substance into a cell.

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BACKGROUND ART

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Techniques for introducing a target substance (e.g., proteins, etc.) into cells (i.e., transfection, transformation, transduction, etc.) are generally used in a wide variety of fields, such as cell biology, genetic engineering, molecular biology, and the like.

20

Transfection is conducted to temporarily express a gene in cells, such as animal cells and the like, so as to observe an influence of the gene. Since the advent of the postgenome era, transfection techniques are frequently used to elucidate the functions of genes encoded by the genome.

25

Various techniques and agents used therein have been developed to achieve transfection. One of the techniques employs a cationic substance, such as a cationic polymer, a cationic lipid, or the like, and is widely used.

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In many cases, however, use of conventional agents

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is not sufficient for transfection efficiency. No agent, which can be used either in solid phase or in liquid phase, has been conventionally developed. Therefore, there is a large demand for such an agent. Further, there is an increasing demand for a technique for efficiently introducing (e.g., transfecting, etc.) a target substance into cells or the like on a solid phase, such as microtiter plates, arrays, and the like.

10 The difficulty in transfecting cells or producing transgenic organisms hinders the progression of development of dominant negative screening in mammals. To overcome this problem, high-efficiency retrovirus transfection has been developed. Although this retrovirus transfection is potent, 15 it is necessary to produce DNA to be packaged into viral intermediates, and therefore, the applicability of this technique is limited. Alternatively, high-density transfection arrays are being developed, but are not necessarily applicable to all cells. Various systems for 20 liquid phase transfection have been developed. However, efficiency is low for adherent cells, for example. Thus, such techniques are not necessarily applicable to all cells.

 Accordingly, a transfection system, which is 25 applicable to all systems and all cells, has been desired in the art. Such a transfection system can be expected to be applied to large-scale high-throughput assays using, for example, microtiter plates, arrays, and the like, for various cells and experimentation systems. There is an increasing 30 demand for such a transfection system.

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide a method for improving the efficiency of introducing (particularly, transfecting) target substances (e.g., DNA, polypeptides, sugars, or complexes thereof, etc.), which are conventionally difficult to introduce into cells via diffusion or hydrophobic interaction, in any circumstances.

The above-described object of the present invention was achieved by unexpectedly finding that a system using an actin acting substance can be used to dramatically increase the efficiency of introducing target substances into cells. This achievement is attributed in part to the unexpected finding that extracellular matrix proteins (e.g., fibronectin, vitronectin, laminin, etc.) act on actin.

Therefore, the present invention provides the following.

(1) A composition for increasing the efficiency of introducing a target substance into a cell, comprising:
(a) an actin acting substance.

(2) A composition according to item 1, wherein the actin acting substance may be an extracellular matrix protein or a variant or fragment thereof.

(3) A composition according to item 2, wherein the actin acting substance comprises at least one protein selected from the group consisting of fibronectin, laminin, and vitronectin, or a variant or fragment thereof.

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(4) A composition according to item 1, wherein the actin acting substance comprises:

(a-1) a protein molecule comprising at least amino acids 21 to 241 of SEQ ID NO.: 11 constituting an Fn1 domain, or a variant thereof;

(a-2) a protein molecule having an amino acid sequence set forth in SEQ ID NO.: 2 or 11, or a variant or fragment thereof;

(b) a polypeptide having an amino acid sequence set forth in SEQ ID NO.: 2 or 11 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a biological activity;

(c) a polypeptide encoded by a splice or allelic mutant of a base sequence set forth in SEQ ID NO.: 1;

(d) a polypeptide being a species homolog of the amino acid sequence set forth in SEQ ID NO.: 2 or 11; or

(e) a polypeptide having an amino acid sequence having at least 70% identity to any one of the polypeptides (a-1) to (d), and having a biological activity.

(5) A composition according to item 1, wherein the Fn1 domain comprises amino acids 21 to 577 of SEQ ID NO.: 11.

(6) A composition according to item 1, wherein the protein molecule having the Fn1 domain is fibronectin or a variant or fragment thereof.

(7) A composition according to item 1, further comprising a gene introduction reagent.

(8) A composition according to item 1, wherein the gene introduction reagent is selected from the group consisting

of cationic polymers, cationic lipids, and calcium phosphate.

(9) A composition according to item 1, further comprising a particle.

5

(10) A composition according to item 9, wherein the particle comprises gold colloid.

10 (11) A composition according to item 1, further comprising a salt.

(12) A composition according to item 11, wherein the salt is selected from the group consisting of salts contained in buffers and salts contained in media.

15

(13) A kit for increasing the efficiency of introducing a target substance into a cell, comprising:

(a) a composition comprising an actin acting substance; and

20

(b) a gene introduction reagent.

(14) A composition for increasing the efficiency of introducing a target substance into a cell, comprising:

A) a target substance; and

25

B) an actin acting substance.

(15) A composition according to item 14, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes thereof.

30

(16) A composition according to item 14, wherein the target substance comprises DNA encoding a gene sequence to be

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transfected.

(17) A composition according to item 16, further comprising a gene introduction reagent.

5

(18) A composition according to item 14, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.

10 (19) A composition according to item 14, wherein the composition is provided in liquid phase.

(20) A composition according to item 14, wherein the composition is provided in solid phase.

15

(21) A device for introducing a target substance into a cell, comprising:

A) a target substance; and

B) an actin acting substance,

20 wherein the composition is fixed to a solid phase support.

(22) A device according to item 21, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes thereof.

25

(23) A device according to item 21, wherein the target substance comprises DNA encoding a gene sequence to be transfected.

30

(24) A device according to item 23, further comprising a gene introduction reagent.

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(25) A device according to item 21, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.

5

(26) A device according to item 21, wherein the solid phase support is selected from the group consisting of plates, microwell plates, chips, glass slides, films, beads, and metals.

10

(27) A device according to item 21, wherein the solid phase support is coated with a coating agent.

15

(28) A device according to item 27, wherein the coating agent comprises a substance selected from the group consisting of poly-L-lysine, silane, MAS, hydrophobic fluorine resins, and metals.

20

(29) A method for increasing the efficiency of introducing a target substance into a cell, comprising the steps of:

A) providing the target substance;

B) providing an actin acting substance; and

C) contacting the target substance and the actin acting substance with the cell.

25

(30) A method according to item 29, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes thereof.

30

(31) A method according to item 29, wherein the target substance comprises DNA encoding a gene sequence to be transfected.

(32) A method according to item 31, further comprising providing a gene introduction reagent, wherein the gene introduction reagent is contacted with the cell.

5

(33) A method according to item 29, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.

10

(34) A method according to item 29, wherein the steps are conducted in liquid phase.

(35) A method according to item 29, wherein the steps are conducted in solid phase.

15

(36) A method for increasing the efficiency of introducing a target substance into a cell, comprising the steps of:

I) fixing a composition to a solid support, wherein the composition comprising:

20

A) a target substance; and

B) an actin acting substance; and

II) contacting the cell with the composition on the solid support.

25

(37) A method according to item 36, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes thereof.

30

(38) A method according to item 36, wherein the target substance comprises DNA encoding a gene sequence to be transfected.

(39) A method according to item 38, further comprising providing a gene introduction reagent, wherein the gene introduction reagent is contacted with the cell.

5 (40) A method according to item 39, further comprising forming a complex of the DNA and the gene introduction reagent after providing the gene introduction reagent, wherein after the forming step, the composition is provided by providing the actin acting substance.

10

(41) A method according to item 36, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.

15

Hereinafter, the present invention will be described by way of preferred embodiments. It will be understood by those skilled in the art that the embodiments of the present invention can be appropriately made or carried out based on the description of the present specification and the
20 accompanying drawings, and commonly used techniques well known in the art. The function and effect of the present invention can be easily recognized by those skilled in the art.

25

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 shows the results of experiments in which various actin acting substances and HEK293 cells were used, where gelatin was used as a control. Figure 1 shows an effect of each adhered substance (HEK cell) with respect to transfection efficiency. The HEK cells were transfected with pEGFP-N1 using an Effectene reagent.

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Figure 2 shows exemplary transfection efficiency when fibronectin fragments were used.

Figure 3 shows exemplary transfection efficiency when fibronectin fragments were used.

Figure 4 shows a summary of the results presented in Figures 2 and 3.

Figure 5 shows the results of an example in which transfection efficiency was studied for various cells.

Figure 6 shows the results of transfection when various plates were used.

Figure 7 shows the results of transfection when various plates were used at a fibronectin concentration of 0, 0.27, 0.53, 0.8, 1.07, and 1.33 ($\mu\text{g}/\mu\text{L}$ for each). Figure 7 shows the influence of fibronectin concentration and the surface modification on the transfection of HEK293 cells. The data shows the average of 4 different squares.

Figure 8 shows exemplary photographs showing cell adhesion profiles in the presence or absence of fibronectin.

Figure 9 shows exemplary cross-sectional photographs of cell adhesion profiles in the presence or absence of fibronectin. Cross-sections of human mesenchymal stem cells (hMSC) were observed using a confocal laser scanning microscope. hMSCs were stained with SYTO61 (blue fluorescence) and Texas red - X phalloidin (red fluorescence) and fixed with 4% PFA. Blue fluorescence (nuclei: SYTO61) and red fluorescence (nuclei: Texas red

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- X phalloidin) were obtained using a confocal laser microscope (LSM510, Carl Zeiss Co., Ltd., pin hole size=1.0, image interval=0.4).

5

Figure 10 shows transition of nuclear surface area. Relative nuclear surface area was determined by cross-sections of hMSC observed with cofocal laser scanning microscopy. hMSC was fixed with 4% PFA.

10

Figure 11 shows the results of an exemplary transfection experiment when a transfection array chip was constructed and used.

15

Figure 12 shows exemplary contamination between each spot on an array.

20

Figures 13A and 13B show an experiment in which spatially-spaced DNA was caused to be taken into cells by the solid phase transfection of the present invention in Example 4. Figure 13A schematically shows a method for producing a solid phase transfection array (SPTA). Figure 13B shows the results of solid phase transfection. A HEK293 cell line was used to produce a SPTA. Green colored portions indicate transfected adherent cells. According to this result, the method of the present invention can be used to produce a group of cells separated spatially and transfected with different genes.

25

30

Figure 13C shows a difference between conventional liquid phase transfection and SPTA.

Figures 14A and 14B shows the results of comparison

of liquid phase transfection and SPTA. Figure 14A shows the results of experiments where 5 cell lines were measured with respect to GFP intensity/mm². Transfection efficiency was determined as fluorescence intensity per unit area.

5 Figure 14B shows fluorescence images of cells expressing EGFP corresponding to the data presented in Figure 14A. White circular regions were regions in which plasmid DNA was fixed. In other regions, cells were also fixed in solid phase, however, cells expressing EGFP were not observed.

10 The white bar indicates 500 μ m.

Figure 14C shows an exemplary transfection method of the present invention.

15 Figure 14D shows an exemplary transfection method of the present invention.

Figures 15A and 15B show the results of coating a chip, where by cross contamination was reduced. Figures 15A and 15B show the results of liquid phase transfection and SPTA using HEK293 cells, HeLa cells, NIT3T3 cells (also referred to as "3T3"), HepG2 cells, and hMSCs. Transfection efficiency was represented by GFP intensity.

20

Figures 16A and 16B show cross contamination between each spot. A nucleic acid mixture containing fibronectin having a predetermined concentration was fixed to a chip coated with APS (γ -aminopropyl silane) or PLL (poly-L-lysine). Cell transfection was performed on the chip. Substantially no cross contamination was observed (upper and middle rows). In contrast, significant chip cross contamination of fixed nucleic acids was observed on a uncoated chip (lower row).

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Figures 16C and 16D show a correlation relationship between the types of substances contained in a mixture used for fixation of nucleic acid and the cell adhesion rate. The graph of Figure 16D shows an increase in the proportion of adherent cells over time. A longer time is required for cell adhesion when the slope of the graph is mild than when the slope of the graph is steep.

Figure 17 shows the results of transfection using an RNAi transfection array of Example 5. Each reporter gene was printed on a solid phase substrate at a rate of 4 points per gene. The substrate was dried. For each transcription factor, siRNA (28 types) were printed onto coordinates at which reporter genes were printed, followed by drying. As a control, siRNA for EGFP was used. As a negative control, scramble RNA was used. Thereafter, LipofectAMINE2000 was printed onto the same coordinates of each gene, followed by drying. Thereafter, fibronectin solution was printed onto the same coordinates of each gene. HeLa-K cells were plated on the substrate, followed by culture for 2 days. Thereafter, images were taken using a fluorescence image scanner.

Figures 18A to 18E show the results of transfection using the RNAi transfection array of Example 5 for each cell. The fluorescence intensity of each reporter was quantified by image analysis, and thereafter, compared with the intensity of each reporter gene to which scramble RNA (negative control) was printed, thereby calculating the ratio. The results are shown for all reporters and all cells. D: pDsRed2-1 (promoterless vector: negative control to shRNA). G: pEGFP-N1 (green fluorescent protein expression vector: a target gene for shRNA used herein). sh: pPUR6iGFP272

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(vector type RNAi suppressing the expression of EGFP gene).
D+G, etc.: D was printed before G was printed (the order
of printing is as written). D+G(7:3), etc.: the ratio of
D to G, where the total amount of D and G genes was 2 µg and
5 the ratio of the D gene to the G gene was 7:3.

Figure 19 shows the results of transfection using
an RNAi transfection array of Example 5. Each reporter gene
expression unit PCR fragment was printed on a solid phase
10 substrate at a rate of 4 points per gene. The substrate was
dried. For each transcription factor, siRNA (28 types) were
printed onto coordinates at which reporter genes were printed,
followed by drying. As a control, siRNA for EGFP was used.
As a negative control, scramble RNA was used. Thereafter,
15 LipofectAMINE2000 was printed onto the same coordinates of
each gene, followed by drying. Thereafter, fibronectin
solution was printed onto the same coordinates of each gene.
HeLa-K cells were plated on the substrate, followed by culture
for 2 days. Thereafter, images were taken using a
20 fluorescence image scanner.

Figures 20A to 20D show the results of transfection
using the RNAi transfection array of Example 6 for each cell.
The fluorescence intensity of each reporter was quantified
25 by image analysis, and thereafter, compared with the
intensity of each reporter gene to which scramble RNA
(negative control) was printed, thereby calculating the ratio.
The results are shown for all reporters and all cells.

30 Figure 21 shows a structure of a PCR fragment obtained
in Example 7.

Figure 22 shows a structure of pEGFP-N1.

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Figure 23 shows the result of comparison of transfection efficiency of transfection microarrays using cyclic DNA and PCR fragments.

5

Figure 24 shows changes when a tetracycline dependent promoter was used.

Figure 25 shows the results of expression when a tetracycline dependent promoter and a tetracycline independent promoter were used.

DESCRIPTION OF SEQUENCE LISTING

15 SEQ ID NO.: 1: a nucleic acid sequence of fibronectin
 (human)
 SEQ ID NO.: 2: an amino acid sequence of fibronectin
 (human)
 SEQ ID NO.: 3: a nucleic acid sequence of vitronectin
20 (mouse)
 SEQ ID NO.: 4: an amino acid sequence of vitronectin
 (mouse)
 SEQ ID NO.: 5: a nucleic acid sequence of laminin
 (mouse α -chain)
25 SEQ ID NO.: 6: an amino acid sequence of laminin
 (mouse α -chain)
 SEQ ID NO.: 7: a nucleic acid sequence of laminin
 (mouse β -chain)
 SEQ ID NO.: 8: an amino acid sequence of laminin
30 (mouse β -chain)
 SEQ ID NO.: 9: a nucleic acid sequence of laminin
 (mouse γ -chain)
 SEQ ID NO.: 10: an amino acid sequence of laminin

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(mouse γ -chain)

SEQ ID NO.: 11: an amino acid sequence of fibronectin

(bovine)

SEQ ID NO.: 12: primer 1 used in Example 7

5 SEQ ID NO.: 13: primer 2 used in Example 7

SEQ ID NO.: 14: a PCR fragment obtained in a PCR
reaction in Example 7

SEQ ID NO.: 15: pTet-Off used in Example 9

SEQ ID NO.: 16: pTet-On used in Example 9

10 SEQ ID NO.: 17: 5 amino acids of laminin

SEQ ID NO.: 18: pTRE-d2EGFP used in Example 9

BEST MODE FOR CARRYING OUT THE INVENTION

15 It should be understood throughout the present
specification that articles for singular forms include the
concept of their plurality unless otherwise mentioned.
Therefore, articles or adjectives for singular forms (e.g.,
"a", "an", "the", etc. in English; "ein", "der", "das", "die",
20 etc. and their inflections in German; "un", "une", "le",
"la", etc. in French; "un", "una", "el", "la", etc. in Spanish,
and articles, adjectives, etc. in other languages) include
the concept of their plurality unless otherwise specified.
It should be also understood that terms as used herein have
25 definitions ordinarily used in the art unless otherwise
mentioned. Therefore, all technical and scientific terms
used herein have the same meanings as commonly understood
by those skilled in the art. Otherwise, the present
application (including definitions) takes precedence.

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(Definition of terms)

Hereinafter, terms specifically used herein will be
defined.

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(Actin acting substances)

As used herein, the term "actin acting substance" refers to a substance which interacts directly or indirectly with actin within cells to alter the form or state of actin. Examples of such a substance include, but are not limited to, extracellular matrix proteins (e.g., fibronectin, vitronectin, laminin, etc.), and the like. Such actin acting substances include substances identified by the following assays. As used herein, interaction with actin is evaluated by visualizing actin with an actin staining reagent (Molecular Probes, Texas Red-X phalloidin) or the like, followed by microscopic inspection to observe and determine actin aggregation, actin reconstruction or an improvement in cellular outgrowth rate. Such evaluation may be performed quantitatively or qualitatively. Actin acting substances are herein utilized so as to increase transfection efficiency. An actin acting substance used herein is derived from any organism, including, for example, mammals, such as human, mouse, bovine, and the like.

As used herein, the term "extracellular matrix protein" refers to a protein constituting an "extracellular matrix". As used herein, the term "extracellular matrix" (ECM) is also called "extracellular substrate" and has the same meaning as commonly used in the art, and refers to a substance existing between somatic cells no matter whether the cells are epithelial cells or non-epithelial cells. Extracellular matrices are involved in supporting tissue as well as in internal environmental structures essential for survival of all somatic cells. Extracellular matrices are generally produced from connective tissue cells. Some extracellular matrices are secreted from cells possessing basal membrane, such as epithelial cells or endothelial cells.

Extracellular matrices are roughly divided into fibrous components and matrices filling there between. Fibrous components include collagen fibers and elastic fibers. A basic component of matrices is glycosaminoglycan (acidic mucopolysaccharide), most of which is bound to non-collagenous protein to form a polymer of a proteoglycan (acidic mucopolysaccharide-protein complex). In addition, matrices include glycoproteins, such as laminin of basal membrane, microfibrils around elastic fibers, fibers, fibronectins on cell surfaces, and the like. Particularly differentiated tissue has the same basic structure. For example, in hyaline cartilage, chondroblasts characteristically produce a large amount of cartilage matrices including proteoglycans. In bones, osteoblasts produce bone matrices which cause calcification. Examples of extracellular matrices for use in the present invention include, but are not limited to, collagen, elastin, proteoglycan, glycosaminoglycan, fibronectin, laminin, elastic fiber, collagen fiber, and the like. An extracellular matrix protein used in the present invention includes, for example, without limitation, fibronectin, vitronectin, laminin, and the like.

Examples of extracellular matrix proteins used in the present invention include, but are not limited to, at least one protein selected from the group consisting of fibronectin and its variants (e.g., pronectin F, pronectin L, pronectin Plus, etc.), laminin, and vitronectin, or a variant or fragment thereof. Such a fragment preferably has a molecular weight of, for example, at least 10 kDa. If a fragment has such a preferable molecular weight and has only 3 amino acids (e.g., a sequence of RGD), preferably at least 5 amino acids (IKVAV, SEQ ID NO.: 17), of an extracellular

matrix protein sequence, the rest of the sequence may be arbitrarily changed as long as the capability of interacting with actin is retained.

5 As used herein, the term "Fn1 domain" typically refers to a sequence of fibronectin extending from the N terminus of its amino acid sequence and having a molecular weight of about 29 kDa (e.g., amino acids 21 to 241 of SEQ ID NO.: 11). In another embodiment, the domain may comprise a sequence
10 of fibronectin extending from the N terminus of its amino acid sequence and having a molecular weight of about 72 kDa (e.g., amino acids 21 to 577 of SEQ ID NO.: 11). As an exemplary actin acting substance of the present invention, a polypeptide comprising the Fn1 domain or a variant thereof
15 may be illustrated without limitation.

 As used herein, the term "fibronectin" has the same meaning as that commonly understood by those skilled in the art, and refers to a protein which is conventionally
20 categorized as an adhesion factor. Attention has been focused onto the cell adhesion function of fibronectin, so that fibronectin is being actively studied.

 A gene encoding fibronectin herein comprises:
25 (a) a polynucleotide having a base sequence set forth in SEQ ID NO.: 1, or a fragment thereof;
 (b) a polynucleotide encoding a polypeptide consisting of an amino acid sequence set forth in SEQ ID NO.: 2 or 11, or a fragment thereof;
30 (c) a polynucleotide encoding a variant polypeptide having the amino acid sequence set forth in SEQ ID NO.: 2 or 11 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition,

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and deletion and having a biological activity;

(d) a polynucleotide which is a splice or allelic mutant of the base sequence set forth in SEQ ID NO.: 1;

(e) a polynucleotide encoding a polypeptide, which
5 is a species homolog of the amino acid sequence set forth in SEQ ID NO.: 2 or 11; or

(g) a polynucleotide consisting of an amino acid sequence having at least 70% identity to any one of the polynucleotides (a) to (e) or a complementary sequence
10 thereof, and encoding a polypeptide having a biological activity. Examples of biological activities include, but are not limited to, cell adhesion activity, heparin binding activity, collagen binding activity, actin acting activity first discovered in the present invention, and the like.
15 A preferable biological activity is actin acting activity.

As used herein, "fibronectin" or "fibronectin polypeptide" comprises:

(a) a protein molecule having at least an amino acid
20 sequence set forth in SEQ ID NO.: 2 or 11, or a variant thereof;

(b) a polypeptide having an amino acid sequence set forth in SEQ ID NO.: 2 or 11 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a
25 biological activity;

(c) a polypeptide encoded by a splice or allelic mutant of a base sequence set forth in SEQ ID NO.: 1;

(d) a polypeptide being a species homolog of the amino acid sequence set forth in SEQ ID NO.: 2 or 11; or

(e) a polypeptide having an amino acid sequence
30 having at least 70% identity to any one of the polypeptides (a) to (d), and having a biological activity.

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As used herein, the term "vitronectin" has the same meaning as that commonly understood by those skilled in the art, and refers to a protein which is conventionally categorized into adhesion factors. Attention has been focused onto the cell adhesion function of vitronectin, so that vitronectin is being actively studied.

As used herein, a gene encoding vitronectin comprises:

- 10 (a) a polynucleotide having a base sequence set forth in SEQ ID NO.: 3, or a fragment thereof;
- (b) a polynucleotide encoding a polypeptide consisting of an amino acid sequence set forth in SEQ ID NO.: 4, or a fragment thereof;
- 15 (c) a polynucleotide encoding a variant polypeptide having the amino acid sequence set forth in SEQ ID NO.: 4 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a biological activity;
- 20 (d) a polynucleotide which is a splice or allelic mutant of the base sequence set forth in SEQ ID NO.: 3;
- (e) a polynucleotide encoding a species homolog of the polypeptide consisting of the amino acid sequence of SEQ ID NO.: 4;
- 25 (f) a polynucleotide hybridizable to any one of the polynucleotides (a) to (e) and encoding a polypeptide having a biological activity; or
- (g) a polynucleotide consisting of a base sequence having at least 70% identity to any one of the polynucleotides (a) to (e) or a complementary sequence thereof, and encoding a polypeptide having a biological activity. Examples of biological activities include, but are not limited to, cell adhesion activity, heparin binding activity, collagen
- 30

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binding activity, complement activating activity, actin acting activity first discovered in the present invention, and the like. A preferable biological activity is actin acting activity.

5

As used herein, "vitronectin" or "vitronectin polypeptide" comprises:

- (a) a protein molecule having at least an amino acid sequence set forth SEQ ID NO.: 4, or a variant thereof;
- 10 (b) a polypeptide having the amino acid sequence set forth in SEQ ID NO.: 4 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a biological activity;
- 15 (c) a polypeptide encoded by a splice or allelic mutant of a base sequence set forth in SEQ ID NO.: 3;
- (d) a polypeptide which is a species homolog of the amino acid sequence set forth in SEQ ID NO.: 4; or
- (e) a polypeptide having an amino acid sequence
20 having at least 70% identity to any one of the polypeptides (a) to (d), and having a biological activity.

As used herein, the term "laminin" has the same meaning as that commonly understood by those skilled in the art, and refers to a protein which is conventionally
25 categorized into adhesion factors. Attention has been focused onto the cell adhesion function of laminin, so that laminin is being actively studied.

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As used herein, a gene encoding laminin comprises:

- (a) polynucleotides having a base sequence set forth in SEQ ID NOS.: 5, 7, and 9, or a fragment thereof;
- (b) polynucleotides encoding a polypeptide

consisting of an amino acid sequence set forth in SEQ ID NOS.: 6, 8, and 10, or a fragment thereof;

(c) polynucleotides encoding a variant polypeptide having the amino acid sequence set forth in SEQ ID NOS.: 6, 8, and 10 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a biological activity;

(d) polynucleotides which are splice or allelic mutants of the base sequence set forth in SEQ ID NOS.: 5, 7, and 9;

(e) polynucleotides encoding a species homolog of a polypeptide consisting of the amino acid sequence set forth in SEQ ID NOS.: 6, 8, and 10;

(f) a polynucleotide hybridizable to any one of the polynucleotides (a) to (e) under stringent conditions, and having a biological activity; or

(g) a polynucleotide consisting of a base sequence having at least 70% identity to any one of the polynucleotides (a) to (e) or a complementary sequence thereof, and encoding a polypeptide having a biological activity. Examples of biological activities include, but are not limited to, cell adhesion activity, heparin binding activity, collagen binding activity, complement activating activity, actin acting activity first discovered in the present invention, and the like. A preferable biological activity is actin acting activity.

As used herein, "laminin" or "laminin polypeptide" comprises:

(a) protein molecules having at least an amino acid sequence set forth in SEQ ID NOS.: 6, 8 and 10, or a variant thereof;

(b) polypeptides having the amino acid sequence set

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forth in SEQ ID NOS.: 6, 8 and 10 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a biological activity;

5 (c) polypeptides encoded by a splice or allelic mutant of a base sequence set forth in SEQ ID NOS.: 5, 7 and 9;

(d) polypeptides which are a species homolog of the amino acid sequence set forth in SEQ ID NOS.: 6, 8 and 10; or

10 (e) a polypeptide having an amino acid sequence having at least 70% identity to any one of the polypeptides (a) to (d), and having a biological activity.

As used herein, the terms "cell adhesion molecule" and "adhesion molecule" are used interchangeably to refer to a molecule capable of mediating the joining of two or more cells (cell adhesion) or adhesion between a substrate and a cell. In general, cell adhesion molecules are divided into two groups: molecules involved in cell-cell adhesion (intercellular adhesion) (cell-cell adhesion molecules) and molecules involved in cell-extracellular matrix adhesion (cell-substrate adhesion) (cell-substrate adhesion molecules). In the method of the present invention, any molecule may be useful and may be effectively used. Therefore, cell adhesion molecules herein include a protein of a substrate and a protein of a cell (e.g., integrin, etc.) in cell-substrate adhesion. A molecule other than proteins falls within the concept of cell adhesion molecule as long as it can mediate cell adhesion.

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For cell-cell adhesion, cadherin, a number of molecules belonging in an immunoglobulin superfamily (NCAM1, ICAM, fasciclin II, III, etc.), selectin, and the like are

- 25 -

known, each of which is known to join cell membranes via a specific molecular reaction.

On the other hand, a major cell adhesion molecule functioning for cell-substrate adhesion is integrin, which recognizes and binds to various proteins contained in extracellular matrices. These cell adhesion molecules are all located on cell membranes and can be regarded as a type of receptor (cell adhesion receptor). Therefore, receptors present on cell membranes can also be used in a method of the present invention. Examples of such a receptor include, but are not limited to, α -integrin, β -integrin, CD44, syndecan, aggrecan, and the like. Techniques for cell adhesion are well known as described above and as described in, for example, "Saibogaimatorikkusu -Rinsho heno Oyo-[Extracellular matrix -Clinical Applications-], Medical Review.

It can be determined whether or not a certain molecule is a cell adhesion molecule, by an assay, such as biochemical quantification (an SDS-PAGE method, a labeled-collagen method, etc.), immunological quantification (an enzyme antibody method, a fluorescent antibody method, an immunohistological study, etc.), a PCR method, a hybridization method, or the like, in which a positive reaction is detected. Examples of such a cell adhesion molecule include, but are not limited to, collagen, integrin, fibronectin, laminin, vitronectin, fibrinogen, an immunoglobulin superfamily member (e.g., CD2, CD4, CD8, ICM1, ICAM2, VCAM1), selectin, cadherin, and the like. Most of these cell adhesion molecules transmit into a cell an auxiliary signal for cell activation due to intercellular interaction as well as cell adhesion. Therefore, an adhesion factor for use in the present invention

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preferably transmits an auxiliary signal for cell activation into a cell. It can be determined whether or not such an auxiliary signal can be transmitted into a cell, by an assay, such as biochemical quantification (an SDS-PAG method, a
5 labeled-collagen method, etc.), immunological quantification (an enzyme antibody method, a fluorescent antibody method, an immunohistological study, etc.), a PDR method, a hybridization method, or the like, in which a positive reaction is detected.

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An example of a cell adhesion molecule is cadherin which is present in many cells capable of being fixed to tissue. Cadherin can be used in a preferred embodiment of the present invention. Examples of a cell adhesion molecule
15 in cells of blood and the immune system which are not fixed to tissue, include, but are not limited to, immunoglobulin superfamily molecules (CD 2, LFA-3, ICAM-1, CD2, CD4, CD8, ICM1, ICAM2, VCAM1, etc.); integrin family molecules (LFA-1, Mac-1, gpIIbIIIa, p150, p95, VLA1, VLA2, VLA3, VLA4, VLA5,
20 VLA6, etc.); selectin family molecules (L-selectin, E-selectin, P-selectin, etc.), and the like. Prior to the disclosure of the present invention, it had not been known that these substances increase transfection efficiency.

25

(General techniques)

Molecular biological techniques, biochemical techniques, and microorganism techniques as used herein are well known in the art and commonly used, and are described in, for example, Sambrook J. et al. (1989), Molecular Cloning:
30 A Laboratory Manual, Cold Spring Harbor and its 3rd Ed. (2001); Ausubel, F.M. (1987), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-interscience; Ausubel, F.M. (1989), Short Protocols in Molecular Biology: A Compendium

of Methods from Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience; Innis, M.A. (1990), PCR Protocols: A Guide to Methods and Applications, Academic Press; Ausubel, F.M. (1992), Short Protocols in
5 Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Greene Pub. Associates; Ausubel, F.M. (1995), Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Greene Pub. Associates; Innis, M.A. et al. (1995),
10 PCR Strategies, Academic Press; Ausubel, F.M. (1999), Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Wiley, and annual updates; Sninsky, J.J. et al. (1999), PCR Applications: Protocols for Functional Genomics, Academic Press; Special
15 issue, Jikken Igaku [Experimental Medicine] "Idenshi Donyu & Hatsugenkaiseki Jikkenho [Experimental Method for Gene Introduction & Expression Analysis]", Yodo-sha, 1997; and the like. Relevant portions (or possibly the entirety) of each of these publications are herein incorporated by
20 reference.

DNA synthesis techniques and nucleic acid chemistry for preparing artificially synthesized genes are described in, for example, Gait, M.J. (1985), Oligonucleotide
25 Synthesis: A Practical Approach, IRL Press; Gait, M.J. (1990), Oligonucleotide Synthesis: A Practical Approach, IRL Press; Eckstein, F. (1991), Oligonucleotides and Analogues: A Practical Approach, IRL Press; Adams, R.L. et al. (1992), The Biochemistry of the Nucleic Acids, Chapman & Hall;
30 Shabarova, Z. et al. (1994), Advanced Organic Chemistry of Nucleic Acids, Weinheim; Blackburn, G.M. et al. (1996), Nucleic Acids in Chemistry and Biology, Oxford University Press; Hermanson, G.T. (1996), Bioconjugate Techniques,

Academic Press; and the like, related portions of which are herein incorporated by reference.

(Definition of terms)

5 Hereinafter, terms specifically used herein will be defined.

As used herein, the term "biological molecule" refers to a molecule relating to an organism and an aggregation thereof. As used herein, the term "biological" or "organism" refers to a biological organism, including, but being not limited to, an animal, a plant, a fungus, a virus, and the like. A biological molecule includes a molecule extracted from an organism and an aggregation thereof, though the present invention is not limited to this. Any molecule capable of affecting an organism and an aggregation thereof fall within the definition of a biological molecule. Therefore, low molecular weight molecules (e.g., low molecular weight molecule ligands, etc.) capable of being used as medicaments fall within the definition of biological molecule as long as an effect on an organism is intended. Examples of such a biological molecule include, but are not limited to, a protein, a polypeptide, an oligopeptide, a peptide, a polynucleotide, an oligonucleotide, a nucleotide, a nucleic acid (e.g., DNA such as cDNA and genomic DNA; RNA such as mRNA), a polysaccharide, an oligosaccharide, a lipid, a low molecular weight molecule (e.g., a hormone, a ligand, an information transmitting substance, a low molecular weight organic molecule, etc.), and a composite molecule thereof (glycolipids, glycoproteins, lipoproteins, etc.), and the like. A biological molecule may include a cell itself or a portion of tissue as long as it is intended to be introduced into a cell. Preferably, a biological molecule may include a nucleic acid (DNA or RNA) or a protein. In another preferred

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embodiment, a biological molecule is a nucleic acid (e.g., genomic DNA or cDNA, or DNA synthesized by PCR or the like). In another preferred embodiment, a biological molecule may be a protein.

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The terms "protein", "polypeptide", "oligopeptide" and "peptide" as used herein have the same meaning and refer to an amino acid polymer having any length. This polymer may be a straight, branched or cyclic chain. An amino acid
10 may be a naturally-occurring or nonnaturally-occurring amino acid, or a variant amino acid. The term may include those assembled into a composite of a plurality of polypeptide chains. The term also includes a naturally-occurring or
15 artificially modified amino acid polymer. Such modification includes, for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification (e.g., conjugation with a labeling moiety). This definition encompasses a polypeptide containing at least one amino acid analog (e.g.,
20 nonnaturally-occurring amino acid, etc.), a peptide-like compound (e.g., peptoid), and other variants known in the art, for example. A gene product, such as an extracellular matrix protein (e.g., fibronectin, etc.), is in the form of a typical polypeptide.

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The terms "polynucleotide", "oligonucleotide", and "nucleic acid" as used herein have the same meaning and refer to a nucleotide polymer having any length. This term also includes an "oligonucleotide derivative" or a
30 "polynucleotide derivative". An "oligonucleotide derivative" or a "polynucleotide derivative" includes a nucleotide derivative, or refers to an oligonucleotide or a polynucleotide having different linkages between

- 30 -

nucleotides from typical linkages, which are interchangeably used. Examples of such an oligonucleotide specifically include 2'-O-methyl-ribonucleotide, an oligonucleotide derivative in which a phosphodiester bond in an oligonucleotide is converted to a phosphorothioate bond, an oligonucleotide derivative in which a phosphodiester bond in an oligonucleotide is converted to a N3'-P5' phosphoroamidate bond, an oligonucleotide derivative in which a ribose and a phosphodiester bond in an oligonucleotide are converted to a peptide-nucleic acid bond, an oligonucleotide derivative in which uracil in an oligonucleotide is substituted with C-5 propynyl uracil, an oligonucleotide derivative in which uracil in an oligonucleotide is substituted with C-5 thiazole uracil, an oligonucleotide derivative in which cytosine in an oligonucleotide is substituted with C-5 propynyl cytosine, an oligonucleotide derivative in which cytosine in an oligonucleotide is substituted with phenoxazine-modified cytosine, an oligonucleotide derivative in which ribose in DNA is substituted with 2'-O-propyl ribose, and an oligonucleotide derivative in which ribose in an oligonucleotide is substituted with 2'-methoxyethoxy ribose. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively-modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be produced by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner et al., Nucleic Acid Res. 19:5081(1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); Rossolini et al., Mol. Cell. Probes 8:91-98(1994)).

A gene for an extracellular matrix protein (e.g., fibronectin, etc.) is in the form of a typical polynucleotide. A polynucleotide may be used for transfection.

5 As used herein, the term "nucleic acid molecule" is used interchangeably with "nucleic acid", "oligonucleotide", and "polynucleotide" and includes cDNA, mRNA, genomic DNA, and the like. As used herein, nucleic acid and nucleic acid molecule may be included by the concept of the term "gene".

10 A nucleic acid molecule encoding the sequence of a given gene includes "splice mutant (variant)". Similarly, a particular protein encoded by a nucleic acid encompasses any protein encoded by a splice variant of that nucleic acid. "Splice mutants", as the name suggests, are products of

15 alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternative) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternative splicing of

20 exons. Alternative polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition. Therefore, extracellular

25 matrix proteins as used herein, which are useful as, for example, actin acting substances, may include their splice mutants.

 As used herein, the term "gene" refers to an element

30 defining a genetic trait. A gene is typically arranged in a given sequence on a chromosome. A gene which defines the primary structure of a protein is called a structural gene. A gene which regulates the expression of a structural gene

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is called a regulatory gene (e.g., promoter). Genes herein include structural genes and regulatory genes unless otherwise specified. Therefore, a fibronectin gene typically includes both a structural gene for fibronectin and a promoter for fibronectin. As used herein, "gene" may refer to "polynucleotide", "oligonucleotide", "nucleic acid", and "nucleic acid molecule" and/or "protein", "polypeptide", "oligopeptide" and "peptide". As used herein, "gene product" includes "polynucleotide", "oligonucleotide", "nucleic acid" and "nucleic acid molecule" and/or "protein", "polypeptide", "oligopeptide" and "peptide", which are expressed by a gene. Those skilled in the art understand what a gene product is, according to the context.

15

As used herein, the term "homology" in relation to a sequence (e.g., a nucleic acid sequence, an amino acid sequence, etc.) refers to the proportion of identity between two or more gene sequences. Therefore, the greater the homology between two given genes, the greater the identity or similarity between their sequences. Whether or not two genes have homology is determined by comparing their sequences directly or by a hybridization method under stringent conditions. When two gene sequences are directly compared with each other, these genes have homology if the DNA sequences of the genes have representatively at least 50% identity, preferably at least 70% identity, more preferably at least 80%, 90%, 95%, 96%, 97%, 98%, or 99% identity with each other. As used herein, the term "similarity" in relation to a sequence (e.g., a nucleic acid sequence, an amino acid sequence, or the like) refers to the proportion of identity between two or more sequences when conservative substitution is regarded as positive

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(identical) in the above-described homology. Therefore, homology and similarity differ from each other in the presence of conservative substitutions. If no conservative substitutions are present, homology and similarity have the same value.

The similarity, identity and homology of amino acid sequences and base sequences are herein compared using BLAST (sequence analyzing tool) with the default parameters.

As used herein, the term "amino acid" may refer to a naturally-occurring or nonnaturally-occurring amino acid as long as the object of the present invention is satisfied. The term "amino acid derivative" or "amino acid analog" refers to an amino acid which is different from a naturally-occurring amino acid and has a function similar to that of the original amino acid. Such amino acid derivatives and amino acid analogs are well known in the art.

The term "naturally-occurring amino acid" refers to an L-isomer of a naturally-occurring amino acid. The naturally-occurring amino acids are glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine, γ -carboxyglutamic acid, arginine, ornithine, and lysine. Unless otherwise indicated, all amino acids as used herein are L-isomers. An embodiment using a D-isomer of an amino acid falls within the scope of the present invention. The term "nonnaturally-occurring amino acid" refers to an amino acid which is ordinarily not found in nature. Examples of nonnaturally-occurring amino acids include D-form of amino acids as described above, norleucine,

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para-nitrophenylalanine, homophenylalanine,
para-fluorophenylalanine, 3-amino-2-benzyl propionic acid,
D- or L-homoarginine, and D-phenylalanine. The term "amino
acid analog" refers to a molecule having a physical property
5 and/or function similar to that of amino acids, but is not
an amino acid. Examples of amino acid analogs include, for
example, ethionine, canavanine, 2-methylglutamine, and the
like. An amino acid mimic refers to a compound which has
a structure different from that of the general chemical
10 structure of amino acids but which functions in a manner
similar to that of naturally-occurring amino acids.

Amino acids may be referred to herein by either their
commonly known three letter symbols or by the one-letter
15 symbols recommended by the IUPAC-IUB Biochemical
Nomenclature Commission. Nucleotides, likewise, may be
referred to by their commonly accepted single-letter codes.

As used herein, the term "corresponding" amino acid
20 or nucleic acid refers to an amino acid or nucleotide in
a given polypeptide or polynucleotide molecule, which has,
or is anticipated to have, a function similar to that of
a predetermined amino acid or nucleotide in a polypeptide
or polynucleotide as a reference for comparison.
25 Particularly, in the case of enzyme molecules, the term refers
to an amino acid which is present at a similar position in
an active site and similarly contributes to catalytic
activity. For example, the Fn1 domain used in the present
invention may be a portion (domain) in an ortholog
30 corresponding to a molecule (fibronectin) containing the
domain.

As used herein, the term "nucleotide" may be either

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naturally-occurring or nonnaturally-occurring. The term "nucleotide derivative" or "nucleotide analog" refers to a nucleotide which is different from naturally-occurring nucleotides and has a function similar to that of the original
5 nucleotide. Such nucleotide derivatives and nucleotide analogs are well known in the art. Examples of such nucleotide derivatives and nucleotide analogs include, but are not limited to, phosphorothioate, phosphoramidate, methylphosphonate, chiral-methylphosphonate, 2-O-methyl
10 ribonucleotide, and peptide-nucleic acid (PNA).

As used herein, the term "fragment" with respect to a polypeptide or polynucleotide refer to a polypeptide or polynucleotide having a sequence length ranging from 1 to
15 n-1 with respect to the full length of the reference polypeptide or polynucleotide (of length n). The length of the fragment can be appropriately changed depending on the purpose. For example, in the case of polypeptides, the lower limit of the length of the fragment includes 3, 4, 5, 6,
20 7, 8, 9, 10, 15, 20, 25, 30, 40, 50 or more nucleotides. Lengths represented by integers which are not herein specified (e.g., 11 and the like) may be appropriate as a lower limit. For example, in the case of polynucleotides, the lower limit of the length of the fragment includes 5,
25 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100 or more nucleotides. Lengths represented by integers which are not herein specified (e.g., 11 and the like) may be appropriate as a lower limit. As used herein, the length of polypeptides or polynucleotides can be represented by the number of amino
30 acids or nucleic acids, respectively. However, the above-described numbers are not absolute. The above-described numbers as the upper or lower limit are intended to include some greater or smaller numbers (e.g.,

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±10%), as long as the same function is maintained. For this purpose, "about" may be herein put ahead of the numbers. However, it should be understood that the interpretation of numbers is not affected by the presence or absence of "about" in the present specification. In the present invention, a fragment preferably has a certain size or more (e.g., 5 kDa or more, etc.). Though not wishing to be bound by any theory, it is considered that a certain size is required for a fragment to act as an actin acting substance.

10

As used herein, "polynucleotides hybridizing under stringent conditions" refers to conditions commonly used and well known in the art. Such a polynucleotide can be obtained by conducting colony hybridization, plaque hybridization, Southern blot hybridization, or the like using a polynucleotide selected from the polynucleotides of the present invention. Specifically, a filter on which DNA derived from a colony or plaque is immobilized is used to conduct hybridization at 65°C in the presence of 0.7 to 1.0 M NaCl. Thereafter, a 0.1 to 2-fold concentration SSC (saline-sodium citrate) solution (1-fold concentration SSC solution is composed of 150 mM sodium chloride and 15 mM sodium citrate) is used to wash the filter at 65°C. Polynucleotides identified by this method are referred to as "polynucleotides hybridizing under stringent conditions". Hybridization can be conducted in accordance with a method described in, for example, Molecular Cloning 2nd ed., Current Protocols in Molecular Biology, Supplement 1-38, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995), and the like. Here, sequences hybridizing under stringent conditions exclude, preferably, sequences containing only A or T. "Hybridizable polynucleotide" refers to a polynucleotide which can

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hybridize other polynucleotides under the above-described hybridization conditions. Specifically, the hybridizable polynucleotide includes at least a polynucleotide having a homology of at least 60% to the base sequence of DNA encoding a polypeptide having an amino acid sequence specifically
5 herein disclosed, preferably a polynucleotide having a homology of at least 80%, and more preferably a polynucleotide having a homology of at least 95%.

10 As used herein, the term "salt" has the same meaning as that commonly understood by those skilled in the art, including both inorganic and organic salts. Salts are typically generated by neutralizing reactions between acids and bases. Salts include NaCl, K₂SO₄, and the like, which
15 are generated by neutralization, and in addition, PbSO₄, ZnCl₂, and the like, which are generated by reactions between metals and acids. The latter salts may not be generated directly by neutralizing reactions, but may be regarded as a product of neutralizing reactions between acids and bases. Salts
20 may be divided into the following categories: normal salts (salts without any H of acids or without any OH of bases, including, for example, NaCl, NH₄Cl, CH₃COONa, and Na₂CO₃), acid salts (salts with remaining H of acids, including, for example, NaHCO₃, KHSO₄, and CaHPO₄), and basic salts (salts
25 with remaining OH of bases, including, for example, MgCl(OH) and CuCl(OH)). This classification is not very important in the present invention. Examples of preferable salts include salts constituting medium (e.g., calcium chloride, sodium hydrogen phosphate, sodium hydrogen carbonate, sodium
30 pyruvate, HEPES, sodium chloride, potassium chloride, magnesium sulfide, iron nitrate, amino acids, vitamins, etc.), salts constituting buffer (e.g., calcium chloride, magnesium chloride, sodium hydrogen phosphate, sodium chloride, etc.),

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and the like. These salts are preferable as they have a high affinity for cells and thus are better able to maintain cells in culture. These salts may be used singly or in combination. Preferably, these salts may be used in combination. This
5 is because a combination of salts tends to have a higher affinity for cells. Therefore, a plurality of salts (e.g., calcium chloride, magnesium chloride, sodium hydrogen phosphate, and sodium chloride) are preferably contained in medium, rather than only NaCl or the like. More preferably,
10 all salts for cell culture medium may be added to the medium. In another preferred embodiment, glucose may be added to medium.

As used herein, the term "search" indicates that a
15 given nucleic acid sequence is utilized to find other nucleic acid base sequences having a specific function and/or property either electronically or biologically, or using other methods. Examples of an electronic search include, but are not limited to, BLAST (Altschul et al., J. Mol. Biol.
20 215:403-410 (1990)), FASTA (Pearson & Lipman, Proc. Natl. Acad. Sci., USA 85:2444-2448 (1988)), Smith and Waterman method (Smith and Waterman, J. Mol. Biol. 147:195-197 (1981)), and Needleman and Wunsch method (Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970)), and the like. Examples of
25 a biological search include, but are not limited to, a macroarray in which genomic DNA is attached to a nylon membrane or the like or a microarray (microassay) in which genomic DNA is attached to a glass plate under stringent hybridization, PCR and in situ hybridization, and the like. It will be
30 understood that Fnl includes corresponding genes identified by such an electronic or biological search.

As used herein, the term "introduction" of a substance

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into a cell indicates that the substance enters the cell through the cell membrane. It can be determined whether or not the substance is successfully introduced into the cell, as follows. For example, the substance is labeled (e.g.,
5 with a fluorescent label, a chemoluminescent label, a phosphorescent label, a radioactive label, etc.) and the label is detected. Alternatively, changes in the cell, which are attributed to the substance (e.g., gene expression, signal transduction, events caused by binding to
10 intracellular receptors, changes in metabolism, etc.), are measured physically (e.g., visual inspection, etc.), chemically (e.g., measurement of secreted substances, etc.), biochemically, or biologically. Therefore, the term "introduction" encompasses transfection, transformation,
15 transduction and the like, which are usually called genetic manipulations as well as transferring of substances, such as proteins, into cells.

As used herein, the term "target substance" refers
20 to a substance which is intended to be introduced into cells. Substances targeted by the present invention are substances which are not introduced under normal conditions. Therefore, substances which can be introduced into cells by diffusion or hydrophobic interaction under normal conditions, are not
25 targeted in an important aspect of the present invention. Examples of substances which are not introduced into cells under normal conditions, include, but are not limited to, proteins (polypeptides), RNA, DNA, sugars (particularly, polysaccharides), and composite molecules thereof (e.g.,
30 glycoproteins, PNA, etc.), viral vectors, and other compounds.

As used herein, the term "device" refers to a part

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which can constitute the whole or a portion of an apparatus, and comprises a support (preferably, a solid phase support) and a target substance carried thereon. Examples of such a device include, but are not limited to, chips, arrays, microtiter plates, cell culture plates, Petri dishes, films, beads, and the like.

As used herein, the term "support" refers to a material which can fix a substance, such as a biological molecule. Such a support may be made from any fixing material which has a capability of binding to a biological molecule as used herein via covalent or noncovalent bond, or which may be induced to have such a capability.

Examples of materials used for supports include any material capable of forming a solid surface, such as, without limitation, glass, silica, silicon, ceramics, silicon dioxide, plastics, metals (including alloys), naturally-occurring and synthetic polymers (e.g., polystyrene, cellulose, chitosan, dextran, and nylon), and the like. A support may be formed of layers made of a plurality of materials. For example, a support may be made of an inorganic insulating material, such as glass, quartz glass, alumina, sapphire, forsterite, silicon oxide, silicon carbide, silicon nitride, or the like. A support may be made of an organic material, such as polyethylene, ethylene, polypropylene, polyisobutylene, polyethylene terephthalate, unsaturated polyester, fluorine-containing resin, polyvinyl chloride, polyvinylidene chloride, polyvinyl acetate, polyvinyl alcohol, polyvinyl acetal, acrylic resin, polyacrylonitrile, polystyrene, acetal resin, polycarbonate, polyamide, phenol resin, urea resin, epoxy resin, melamine resin, styrene-acrylonitrile copolymer,

acrylonitrile-butadiene-styrene copolymer, silicone resin, polyphenylene oxide, polysulfone, and the like. Also in the present invention, nitrocellulose film, nylon film, PVDF film, or the like, which are used in blotting, may be used
5 as a material for a support. When a material constituting a support is in the solid phase, such as a support is herein particularly referred to as a "solid phase support". A solid phase support may be herein in the form of a plate, a microwell plate, a chip, a glass slide, a film, beads, a metal (surface),
10 or the like. A support may not be coated or may be coated.

As used herein, the term "liquid phase" has the same meanings as commonly understood by those skilled in the art, typically referring a state in solution.
15

As used herein, the term "solid phase" has the same meanings as commonly understood by those skilled in the art, typically referring to a solid state. As used herein, liquid and solid may be collectively referred to as a "fluid".
20

As used herein, the term "contact" means that two substances (e.g., a compositions and a cell) are sufficiently close to each other so that the two substances interact with each other.
25

As used herein, the term "interaction" refers to, without limitation, hydrophobic interactions, hydrophilic interactions, hydrogen bonds, Van der Waals forces, ionic interactions, nonionic interactions, electrostatic
30 interactions, and the like. Preferably, interaction may be a typical interaction, such as a hydrogen bond, a hydrophobic interaction, or the like, which takes place in organisms.

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(Modification of genes)

An actin acting substance used in the present invention is often used in the form of a gene product. It will be understood that such a gene product may be a variant thereof. Therefore, substances produced using the gene modification techniques described below can be used in the present invention.

In a given protein molecule, a given amino acid may be substituted with another amino acid in a structurally important region, such as a cationic region or a substrate molecule binding site, without a clear reduction or loss of interactive binding ability. A given biological function of a protein is defined by the interactive ability or other property of the protein. Therefore, a particular amino acid substitution may be performed in an amino acid sequence, or at the DNA sequence level, to produce a protein which maintains the original property after the substitution. Therefore, various modifications of peptides as disclosed herein and DNA encoding such peptides may be performed without clear losses of biological activity.

When the above-described modifications are designed, the hydrophobicity indices of amino acids may be taken into consideration. The hydrophobic amino acid indices play an important role in providing a protein with an interactive biological function, which is generally recognized in the art (Kyte, J. and Doolittle, R.F., J. Mol. Biol. 157(1):105-132, 1982). The hydrophobic property of an amino acid contributes to the secondary structure of a protein and then regulates interactions between the protein and other molecules (e.g., enzymes, substrates, receptors, DNA, antibodies, antigens, etc.). Each amino acid is given a

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hydrophobicity index based on the hydrophobicity and charge properties thereof as follows: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8);
5 glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamic acid (-3.5); glutamine (-3.5); aspartic acid (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

10 It is well known that if a given amino acid is substituted with another amino acid having a similar hydrophobicity index, the resultant protein may still have a biological function similar to that of the original protein (e.g., a protein having an equivalent enzymatic activity).
15 For such an amino acid substitution, the hydrophobicity index is preferably within ± 2 , more preferably within ± 1 , and even more preferably within ± 0.5 . It is understood in the art that such an amino acid substitution based on hydrophobicity is efficient. As described in US Patent No. 4,554,101, amino
20 acid residues are given the following hydrophilicity indices: arginine (+3.0); lysine (+3.0); aspartic acid (+3.0 \pm 1); glutamic acid (+3.0 \pm 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 \pm 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0);
25 methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). It is understood that an amino acid may be substituted with another amino acid which has a similar hydrophilicity index and can still provide a biological
30 equivalent. For such an amino acid substitution, the hydrophilicity index is preferably within ± 2 , more preferably ± 1 , and even more preferably ± 0.5 .

The term "conservative substitution" as used herein refers to amino acid substitution in which a substituted amino acid and a substituting amino acid have similar hydrophilicity indices or/and hydrophobicity indices. For example, the conservative substitution is carried out between amino acids having a hydrophilicity or hydrophobicity index of within ± 2 , preferably within ± 1 , and more preferably within ± 0.5 . Examples of the conservative substitution include, but are not limited to, substitutions within each of the following residue pairs: arginine and lysine; glutamic acid and aspartic acid; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine, which are well known to those skilled in the art.

As used herein, the term "variant" refers to a substance, such as a polypeptide, polynucleotide, or the like, which differs partially from the original substance. Examples of such a variant include a substitution variant, an addition variant, a deletion variant, a truncated variant, an allelic variant, and the like. Examples of such a variant include, but are not limited to, a nucleotide or polypeptide having one or several substitutions, additions and/or deletions or a nucleotide or polypeptide having at least one substitution, addition and/or deletion. The term "allele" as used herein refers to a genetic variant located at a locus identical to a corresponding gene, where the two genes are distinguished from each other. Therefore, the term "allelic variant" as used herein refers to a variant which has an allelic relationship with a given gene. Such an allelic variant ordinarily has a sequence the same as or highly similar to that of the corresponding allele, and ordinarily has almost the same biological activity, though it rarely has different biological activity. The term

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"species homolog" or "homolog" as used herein refers to one that has an amino acid or nucleotide homology with a given gene in a given species (preferably at least 60% homology, more preferably at least 80%, at least 85%, at least 90%, and at least 95% homology). A method for obtaining such a species homolog is clearly understood from the description of the present specification. The term "orthologs" (also called orthologous genes) refers to genes in different species derived from a common ancestry (due to speciation). For example, in the case of the hemoglobin gene family having multigene structure, human and mouse α -hemoglobin genes are orthologs, while the human α -hemoglobin gene and the human β -hemoglobin gene are paralogs (genes arising from gene duplication). Orthologs are useful for estimation of molecular phylogenetic trees. Usually, orthologs in different species may have a function similar to that of the original species. Therefore, orthologs of the present invention may be useful in the present invention.

As used herein, the term "conservative (or conservatively modified) variant" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids which encode identical or essentially identical amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For example, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" which represent one species of

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conservatively modified variation. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. Those skilled in the art will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence. Preferably, such modification may be performed while avoiding substitution of cysteine which is an amino acid capable of largely affecting the higher-order structure of a polypeptide. Examples of a method for such modification of a base sequence include cleavage using a restriction enzyme or the like; ligation or the like by treatment using DNA polymerase, Klenow fragments, DNA ligase, or the like; and a site specific base substitution method using synthesized oligonucleotides (specific-site directed mutagenesis; Mark Zoller and Michael Smith, *Methods in Enzymology*, 100, 468-500(1983)). Modification can be performed using methods ordinarily used in the field of molecular biology.

In order to prepare functionally equivalent polypeptides, amino acid additions, deletions, or modifications can be performed in addition to amino acid substitutions. Amino acid substitution(s) refers to the replacement of at least one amino acid of an original peptide with different amino acids, such as the replacement of 1 to 10 amino acids, preferably 1 to 5 amino acids, and more preferably 1 to 3 amino acids with different amino acids. Amino acid addition(s) refers to the addition of at least one amino acid to an original peptide chain, such as the addition of 1 to 10 amino acids, preferably 1 to 5 amino

acids, and more preferably 1 to 3 amino acids to an original peptide chain. Amino acid deletion(s) refers to the deletion of at least one amino acid, such as the deletion of 1 to 10 amino acids, preferably 1 to 5 amino acids, and more preferably 1 to 3 amino acids. Amino acid modification includes, but is not limited to, amidation, carboxylation, sulfation, halogenation, truncation, lipidation, alkylation, glycosylation, phosphorylation, hydroxylation, acylation (e.g., acetylation), and the like. Amino acids to be substituted or added may be naturally-occurring or nonnaturally-occurring amino acids, or amino acid analogs. Naturally-occurring amino acids are preferable.

As used herein, the term "peptide analog" or "peptide derivative" refers to a compound which is different from a peptide but has at least one chemical or biological function equivalent to the peptide. Therefore, a peptide analog includes one that has at least one amino acid analog or amino acid derivative addition or substitution with respect to the original peptide. A peptide analog has the above-described addition or substitution so that the function thereof is substantially the same as the function of the original peptide (e.g., a similar pKa value, a similar functional group, a similar binding manner to other molecules, a similar water-solubility, and the like). Such a peptide analog can be prepared using techniques well known in the art. Therefore, a peptide analog may be a polymer containing an amino acid analog.

Similarly, the term "polynucleotide analog" or "nucleic acid analog" refers to a compound which is different from a polynucleotide or a nucleic acid but has at least one chemical function or biological function equivalent to

that of a polynucleotide or a nucleic acid. Therefore, a polynucleotide analog or a nucleic acid analog includes one that has at least one nucleotide analog or nucleotide derivative addition or substitution with respect to the original peptide.

Nucleic acid molecules as used herein includes one in which a part of the sequence of the nucleic acid is deleted or is substituted with other base(s), or an additional nucleic acid sequence is inserted, as long as a polypeptide expressed by the nucleic acid has substantially the same activity as that of the naturally-occurring polypeptide, as described above. Alternatively, an additional nucleic acid may be linked to the 5' terminus and/or 3' terminus of the nucleic acid. The nucleic acid molecule may include one that is hybridizable to a gene encoding a polypeptide under stringent conditions and encodes a polypeptide having substantially the same function as that of that polypeptide. Such a gene is known in the art and can be used in the present invention.

The above-described nucleic acid can be obtained by a well-known PCR method, i.e., chemical synthesis. This method may be combined with, for example, site-specific mutagenesis, hybridization, or the like.

As used herein, the term "substitution, addition or deletion" for a polypeptide or a polynucleotide refers to the substitution, addition or deletion of an amino acid or its substitute, or a nucleotide or its substitute with respect to the original polypeptide or polynucleotide. This is achieved by techniques well known in the art, including a site-specific mutagenesis technique and the like. A polypeptide or a polynucleotide may have any number (>0)

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of substitutions, additions, or deletions. The number can be as large as a variant having such a number of substitutions, additions or deletions maintains an intended function (e.g., the information transfer function of hormones and cytokines, etc.). For example, such a number may be one or several, and preferably within 20% or 10% of the full length, or no more than 100, no more than 50, no more than 25, or the like.

10 (Interactive agent)

As used herein, the term "agent capable of specifically interacting with" a biological agent, such as a polynucleotide, a polypeptide or the like, refers to an agent which has an affinity to the biological agent, such as a polynucleotide, a polypeptide or the like, which is representatively higher than or equal to an affinity to other non-related biological agents, such as polynucleotides, polypeptides or the like (particularly, those with identity of less than 30%), and preferably significantly (e.g., statistically significantly) higher. Such an affinity can be measured with, for example, a hybridization assay, a binding assay, or the like.

As used herein, the term "agent" may refer to any substance or element as long as an intended object can be achieved (e.g., energy, etc.). Examples of such a substance include, but are not limited to, proteins, polypeptides, oligopeptides, peptides, polynucleotides, oligonucleotides, nucleotides, nucleic acids (e.g., DNA such as cDNA, genomic DNA and the like, or RNA such as mRNA, RNAi and the like), polysaccharides, oligosaccharides, lipids, low molecular weight organic molecules (e.g., hormones, ligands, information transduction substances, low molecular weight

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organic molecules, molecules synthesized by combinatorial chemistry, low molecular weight molecules usable as medicaments (e.g., low molecular weight molecule ligands, etc.), etc.), and composite molecules thereof. Examples of
5 an agent specific to a polynucleotide include, but are not limited to, representatively, a polynucleotide having complementarity to the sequence of the polynucleotide with a predetermined sequence homology (e.g., 70% or more sequence identity), a polypeptide such as a transcriptional agent
10 binding to a promoter region, and the like. Examples of an agent specific to a polypeptide include, but are not limited to, representatively, an antibody specifically directed to the polypeptide or derivatives or analogs thereof (e.g., single chain antibody), a specific ligand or receptor when
15 the polypeptide is a receptor or ligand, a substrate when the polypeptide is an enzyme, and the like.

As used herein, the term "isolated" biological agent (e.g., nucleic acid, protein, or the like) refers to a
20 biological agent that is substantially separated or purified from other biological agents in cells of a naturally-occurring organism (e.g., in the case of nucleic acids, agents other than nucleic acids and a nucleic acid having nucleic acid sequences other than an intended nucleic
25 acid; and in the case of proteins, agents other than proteins and proteins having an amino acid sequence other than an intended protein). The "isolated" nucleic acids and proteins include nucleic acids and proteins purified by a standard purification method. The isolated nucleic acids
30 and proteins also include chemically synthesized nucleic acids and proteins.

As used herein, the term "purified" biological agent

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(e.g., nucleic acids, proteins, and the like) refers to one from which at least a part of naturally accompanying agents is removed. Therefore, ordinarily, the purity of a purified biological agent is higher than that of the biological agent in a normal state (i.e., concentrated).

As used herein, the terms "purified" and "isolated" mean that the same type of biological agent is present preferably at least 75% by weight, more preferably at least 85% by weight, even more preferably at least 95% by weight, and most preferably at least 98% by weight.

(Genetic manipulation)

When genetic manipulation is mentioned herein, the term "vector" or "recombinant vector" refers to a vector transferring a polynucleotide sequence of interest to a target cell. Such a vector is capable of self-replication or incorporation into a chromosome in a host cell (e.g., a prokaryotic cell, yeast, an animal cell, a plant cell, an insect cell, an individual animal, and an individual plant, etc.), and contains a promoter at a site suitable for transcription of a polynucleotide of the present invention. A vector suitable for performing cloning is referred to as a "cloning vector". Such a cloning vector ordinarily contains a multiple cloning site containing a plurality of restriction sites. Restriction enzyme sites and multiple cloning sites as described above are well known in the art and can be used as appropriate by those skilled in the art depending on the purpose in accordance with publications described herein (e.g., Sambrook et al., *supra*).

As used herein, the term "expression vector" refers to a nucleic acid sequence comprising a structural gene and

a promoter for regulating expression thereof, and in addition, various regulatory elements in a state that allows them to operate within host cells. The regulatory element may include, preferably, terminators, selectable markers such as drug-resistance genes, and enhancers.

Examples of "recombinant vectors" for prokaryotic cells include, but are not limited to, pCDNA3(+), pBluescript-SK(+/-), pGEM-T, pEF-BOS, pEGFP, pHAT, pUC18, pFT-DEST™42GATEWAY (Invitrogen), and the like.

Examples of "recombinant vectors" for animal cells include, but are not limited to, pcDNAI/Amp, pcDNAI, pCDM8 (all commercially available from Funakoshi), pAGE107 [Japanese Laid-Open Publication No. 3-229 (Invitrogen), pAGE103 [J. Biochem., 101, 1307(1987)], pAMo, pAMoA [J. Biol. Chem., 268, 22782-22787(1993)], a retrovirus expression vector based on a murine stem cell virus (MSCV), pEF-BOS, pEGFP, and the like.

Examples of recombinant vectors for plant cells include, but are not limited to, pPCVICEn4HPT, pCGN1548, pCGN1549, pBI221, pBI121, and the like.

As used herein, the term "terminator" refers to a sequence which is located downstream of a protein-encoding region of a gene and which is involved in the termination of transcription when DNA is transcribed into mRNA, and the addition of a poly-A sequence. It is known that a terminator contributes to the stability of mRNA, and has an influence on the amount of gene expression.

As used herein, the term "promoter" refers to a base

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sequence which determines the initiation site of transcription of a gene and is a DNA region which directly regulates the frequency of transcription. Transcription is started by RNA polymerase binding to a promoter. A promoter region is usually located within about 2 kbp upstream of the first exon of a putative protein coding region. Therefore, it is possible to estimate a promoter region by predicting a protein coding region in a genomic base sequence using DNA analysis software. A putative promoter region is usually located upstream of a structural gene, but depending on the structural gene, i.e., a putative promoter region may be located downstream of a structural gene. Preferably, a putative promoter region is located within about 2 kbp upstream of the translation initiation site of the first exon. Examples of a promoter include, but are not limited to, a structural promoter, a specific promoter, an inductive promoter, and the like.

As used herein, the term "enhancer" refers to a sequence which is used so as to enhance the expression efficiency of a gene of interest. One or more enhancers may be used, or no enhancer may be used.

As used herein, the term "silencer" refers to a sequence which has a function of suppressing and arresting the expression of a gene. Any silencer which has such a function may be herein used. No silencer may be used.

As used herein, the term "operably linked" indicates that a desired sequence is located such that expression (operation) thereof is under control of a transcription and translation regulatory sequence (e.g., a promoter, an enhancer, and the like) or a translation regulatory sequence.

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In order for a promoter to be operably linked to a gene, typically, the promoter is located immediately upstream of the gene. A promoter is not necessarily adjacent to a structural gene.

5

Any technique may be used herein for introduction of a nucleic acid molecule into cells, including, for example, transformation, transduction, transfection, and the like. Such a nucleic acid molecule introduction technique is well known in the art and commonly used, and is described in, for example, Ausubel F.A. et al., editors, (1988), Current Protocols in Molecular Biology, Wiley, New York, NY; Sambrook J. et al. (1987) Molecular Cloning: A Laboratory Manual, 2nd Ed. and its 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Special issue, Jikken Igaku [Experimental Medicine] "Experimental Method for Gene introduction & Expression Analysis", Yodo-sha, 1997; and the like. Gene introduction can be confirmed by method as described herein, such as Northern blotting analysis and Western blotting analysis, or other well-known, common techniques.

Any of the above-described methods for introducing DNA into cells can be used as a vector introduction method, including, for example, transfection, transduction, transformation, and the like (e.g., a calcium phosphate method, a liposome method, a DEAE dextran method, an electroporation method, a particle gun (gene gun) method, and the like), a lipofection method, a spheroplast method (Proc. Natl. Acad. Sci. USA, 84, 1929(1978)), a lithium acetate method (J. Bacteriol., 153, 163(1983); and Proc. Natl. Acad. Sci. USA, 75, 1929(1978)), and the like.

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As used herein, the term "gene introduction reagent" refers to a reagent which is used in a gene introduction method so as to enhance introduction efficiency. Examples of such a gene introduction reagent include, but are not limited to, cationic polymers, cationic lipids, polyamine-based reagents, polyimine-based reagents, calcium phosphate, and the like. Specific examples of a reagent used in transfection include reagents available from various sources, such as, without limitation, Effectene Transfection Reagent (cat. no. 301425, Qiagen, CA), TransFast™ Transfection Reagent (E2431, Promega, WI), Tfx™-20 Reagent (E2391, Promega, WI), SuperFect Transfection Reagent (301305, Qiagen, CA), PolyFect Transfection Reagent (301105, Qiagen, CA), LipofectAMINE 2000 Reagent (11668-019, Invitrogen corporation, CA), JetPEI (×4) conc. (101-30, Polyplus-transfection, France) and ExGen 500 (R0511, Fermentas Inc., MD), and the like.

As used herein, "instructions" describe a method for introducing a target substance according to the present invention for users (e.g., researchers, laboratory technicians, medical doctors, patients, etc.). The instructions describe a statement indicating a method for using a composition of the present invention, or the like. The instructions are prepared in accordance with a format defined by an authority of a country in which the present invention is practiced (e.g., Health, Labor and Welfare Ministry in Japan, Food and Drug Administration (FDA) in the U.S., and the like), explicitly describing that the instructions are approved by the authority. The instructions are a so-called package insert in the case of medicaments or a manual in the case of experimental reagents, and are typically provided in paper media. The instructions

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are not so limited and may be provided in the form of electronic media (e.g., web sites, electronic mails, and the like provided on the internet).

5 As used herein, the term "transformant" refers to the whole or a part of an organism, such as a cell, which is produced by transformation. Examples of a transformant include a prokaryotic cell, yeast, an animal cell, a plant cell, an insect cell, and the like. Transformants may be referred to as transformed cells, transformed tissue, transformed hosts, or the like, depending on the subject. A cell used herein may be a transformant.

15 When a prokaryotic cell is used herein for genetic operations or the like, the prokaryotic cell may be of, for example, genus *Escherichia*, genus *Serratia*, genus *Bacillus*, genus *Brevibacterium*, genus *Corynebacterium*, genus *Microbacterium*, genus *Pseudomonas*, or the like. Specifically, the prokaryotic cell is, for example, 20 *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, or the like. Alternatively, a cell separated from a naturally-occurring product may be used in the present invention.

25 Examples of an animal cell as used herein include a mouse myeloma cell, a rat myeloma cell, a mouse hybridoma cell, a Chinese hamster ovary (CHO) cell, a baby hamster kidney (BHK) cell, an African green monkey kidney cell, a human leukemic cell, HBT5637 (Japanese Laid-Open Publication 30 No. 63-299), a human colon cancer cell line, and the like. The mouse myeloma cell includes ps20, NSO, and the like. The rat myeloma cell includes YB2/0 and the like. A human embryo kidney cell includes HEK293 (ATCC: CRL-1573) and the

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like. The human leukemic cell includes BALL-1 and the like. The African green monkey kidney cell includes COS-1, COS-7, and the like. The human colon cancer cell line includes, but is not limited to, HCT-15, human neuroblastoma (e.g.,
5 SK-N-SH, SK-N-SH-5Y, etc.), mouse neuroblastoma (e.g., etc.), and the like. Alternatively, primary culture cells may be used in the present invention.

10 Examples of plant cells used herein in genetic manipulation include, but are not limited to, calluses or a part thereof, suspended culture cells, cells of plants in the families of *Solanaceae*, *Poaceae*, *Brassicaceae*, *Rosaceae*, *Leguminosae*, *Cucurbitaceae*, *Lamiaceae*, *Liliaceae*, *Chenopodiaceae* and *Umbelliferae*, and the like.

15 Gene expression (e.g., mRNA expression, polypeptide expression) may be "detected" or "quantified" by an appropriate method, including mRNA measurement and immunological measurement method. Examples of molecular
20 biological measurement methods include Northern blotting methods, dot blotting methods, PCR methods, and the like. Examples of immunological measurement method include ELISA methods, RIA methods, fluorescent antibody methods, Western blotting methods, immunohistological staining methods, and
25 the like, where a microtiter plate may be used. Examples of quantification methods include ELISA methods, RIA methods, and the like. A gene analysis method using an array (e.g., a DNA array, a protein array, etc.) may be used. The DNA array is widely reviewed in Saibo-Kogaku [Cell Engineering],
30 special issue, "DNA Microarray and Up-to-date PCR Method", edited by Shujun-sha. The protein array is described in detail in Nat Genet. 2002 Dec; 32 Suppl:526-32. Examples of methods for analyzing gene expression include, but are

not limited to, RT-PCR methods, RACE methods, SSCP methods, immunoprecipitation methods, two-hybrid systems, *in vitro* translation methods, and the like in addition to the above-described techniques. Other analysis methods are described in, for example, "Genome Analysis Experimental Method, Yusuke Nakamura's Lab-Manual, edited by Yusuke Nakamura, Yodo-sha (2002), and the like. All of the above-described publications are herein incorporated by reference.

As used herein, the term "expression" of a gene, a polynucleotide, a polypeptide, or the like, indicates that the gene or the like is affected by a predetermined action *in vivo* to be changed into another form. Preferably, the term "expression" indicates that genes, polynucleotides, or the like are transcribed and translated into polypeptides. In one embodiment of the present invention, genes may be transcribed into mRNA. More preferably, these polypeptides may have post-translational processing modifications.

As used herein, the term "expression level" refers to the amount of a polypeptide or mRNA expressed in a subject cell. The term "expression level" includes the level of protein expression of a polypeptide evaluated by any appropriate method using an antibody, including immunological measurement methods (e.g., an ELISA method, an RIA method, a fluorescent antibody method, a Western blotting method, an immunohistological staining method, and the like, or the mRNA level of expression of a polypeptide evaluated by any appropriate method, including molecular biological measurement methods (e.g., a Northern blotting method, a dot blotting method, a PCR method, and the like). The term "change in expression level" indicates that an

increase or decrease in the protein or mRNA level of expression of a polypeptide evaluated by an appropriate method including the above-described immunological measurement method or molecular biological measurement method.

5

Therefore, as used herein, the term "reduction" of "expression" of a gene, a polynucleotide, a polypeptide, or the like indicates that the level of expression is significantly reduced in the presence of or under the action of the agent of the present invention as compared to when the action of the agent is absent. Preferably, the reduction of expression includes a reduction in the amount of expression of a polypeptide. As used herein, the term "increase" of "expression" of a gene, a polynucleotide, a polypeptide, or the like indicates that the level of expression is significantly increased by introduction of an agent related to gene expression into cells (e.g., a gene to be expressed or an agent regulating such gene expression) as compared to when the action of the agent is absent. Preferably, the increase of expression includes an increase in the amount of expression of a polypeptide. As used herein, the term "induction" of "expression" of a gene indicates that the amount of expression of the gene is increased by applying a given agent to a given cell. Therefore, the induction of expression includes allowing a gene to be expressed when expression of the gene is not otherwise observed, and increasing the amount of expression of the gene when expression of the gene is observed.

30

As used herein, the term "specifically expressed" in relation to a gene indicates that the gene is expressed in a specific site or for a specific period of time, at a level different from (preferably higher than) that in other

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sites or for other periods of time. The term "specifically expressed" indicates that a gene may be expressed only in a given site (specific site) or may be expressed in other sites. Preferably, the term "specifically expressed" indicates that a gene is expressed only in a given site.

As used herein, the term "biological activity" refers to activity possessed by an agent (e.g., a polynucleotide, a protein, etc.) within an organism, including activities exhibiting various functions (e.g., transcription promoting activity, etc.). For example, when an actin acting substance interacts with actin, the biological activity thereof includes morphological changes in actin (e.g., an increase in cell extending speed, etc.) or other biological changes (e.g., reconstruction of actin filaments, etc.), and the like. Such a biological activity can be measured by, for example, visualizing actin with an actin staining reagent (Molecular Probes, Texas Red-X phalloidin) or the like, followed by microscopic inspection to observe aggregation of actin or cell extension. In another preferred embodiment, such a biological activity may be cell adhesion activity, heparin binding activity, collagen binding activity, or the like. Cell adhesion activity can be measured by, for example, measuring the rate of adhesion of disseminated cells to a solid phase, which is regarded as adhesion activity. Heparin binding activity can be measured by, for example, conducting affinity chromatography using heparin-fixed column or the like to determine whether or not a substance binds to the column. Collagen binding activity can be measured by, for example, conducting affinity chromatography using collagen-fixed column or the like to determine whether or not a substance binds to the column. For example, when a certain agent is an enzyme, the biological activity thereof

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includes enzymatic activity. In another example, when a certain agent is a ligand, the ligand binds to a corresponding receptor. Such binding activity is also biological activity. Such biological activity can be measured using techniques well known in the art (see Molecular Cloning, Current
5 Protocols (*supra*), etc.).

As used herein, the term "particle" refers to a substance which has a certain hardness and a certain size
10 or greater. A particle used in the present invention may be made of a metal or the like. Examples of particles used in the present invention include, but are not limited to, gold colloids, silver colloids, latex colloids, and the like.

As used herein, the term "kit" refers to a unit which typically has two or more sections, at least one of which is used to provide a component (e.g., a reagent, a particle, etc.). When materials are not provided after mixing and are preferably provided to prepare a composition immediately
15 before use, a kit form is preferable. Such a kit preferably comprises instructions which describe how a component (e.g., a reagent, a particle, etc.) should be processed.
20

(Methods for producing polypeptides)

25 A transformant derived from a microorganism, an animal cell, or the like, which possesses a recombinant vector into which DNA encoding a polypeptide of the present invention is incorporated, is cultured according to an ordinary culture method. The polypeptide of the present invention is produced
30 and accumulated. The polypeptide of the present invention is collected from the culture, thereby making it possible to produce the polypeptide of the present invention.

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The transformant of the present invention can be cultured on a culture medium according to an ordinary method for use in culturing host cells. A culture medium for a transformant obtained from a prokaryote (e.g., *E. coli*) or a eukaryote (e.g., yeast) as a host may be either a naturally-occurring culture medium or a synthetic culture medium (e.g., RPMI1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)], 199 medium [Proceedings of the Society for the Biological Medicine, 73, 1 (1950)] or these media supplemented with fetal bovine serum, or the like) as long as the medium contains a carbon source (e.g., carbohydrates (e.g., glucose, fructose, sucrose, molasses containing these, starch, starch hydrolysate, and the like), organic acids (e.g., acetic acid, propionic acid, and the like), alcohols (e.g., ethanol, propanol, and the like), etc.); a nitrogen source (e.g., ammonium salts of inorganic or organic acids (e.g., ammonia, ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, and the like), and other nitrogen-containing substances (e.g., peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysate, soybean cake, and soybean cake hydrolysate, various fermentation bacteria and digestion products thereof), etc.), inorganic salts (e.g., potassium (I) phosphate, potassium (II) phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganous sulfate, copper sulfate, calcium carbonate, etc.), and the like which an organism of the present invention can assimilate and the medium allows efficient culture of the transformant. Culture is performed under aerobic conditions for shaking culture, deep aeration agitation culture, or the like. Culture temperature is preferably 15 to 40°C, culture time is ordinarily 5 hours

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to 7 days. The pH of culture medium is maintained at 3.0 to 9.0. The adjustment of pH is carried out using inorganic or organic acid, alkali solution, urea, calcium carbonate, ammonia, or the like. An antibiotic, such as ampicillin, 5 tetracycline, or the like, may be optionally added to culture medium during cultivation.

A polypeptide of the present invention can be isolated or purified from a culture of a transformant, which has been 10 transformed with a nucleic acid sequence encoding the polypeptide, using an ordinary method for isolating or purifying enzymes, which are well known and commonly used in the art. For example, when a polypeptide of the present invention is secreted outside a transformant for producing 15 the polypeptide, the culture is subjected to centrifugation or the like to obtain a soluble fraction. A purified specimen can be obtained from the soluble fraction by a technique, such as solvent extraction, salting-out/desalting with ammonium sulfate or the like, precipitation with organic 20 solvent, anion exchange chromatography with a resin (e.g., diethylaminoethyl (DEAE)-Sephacrose, DIAION HPA-75 (Mitsubishi Kasei Corporation), etc.), cation exchange chromatography with a resin (e.g., S-Sepharose FF (Pharmacia), etc.), hydrophobic chromatography with a resin (e.g., 25 buthylsepharose, phenylsepharose, etc.), gel filtration with a molecular sieve, affinity chromatography, chromatofocusing, electrophoresis (e.g., isoelectric focusing electrophoresis, etc.).

30 When a polypeptide of the present invention is accumulated in a dissolved form within a transformant cell for producing the polypeptide, the culture is subjected to centrifugation to collect cells in the culture. The cells

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are washed, followed by pulverization of the cells using a ultrasonic pulverizer, a French press, MANTON GAULIN homogenizer, Dinomil, or the like, to obtain a cell-free extract solution. A purified specimen can be obtained from
5 a supernatant obtained by centrifuging the cell-free extract solution or by a technique, such as solvent extraction, salting-out/desalting with ammonium sulfate or the like, precipitation with organic solvent, anion exchange chromatography with a resin (e.g., diethylaminoethyl
10 (DEAE)-Sepharose, DIAION HPA-75 (Mitsubishi Kasei Corporation), etc.), cation exchange chromatography with a resin (e.g., S-Sepharose FF (Pharmacia), etc.), hydrophobic chromatography with a resin (e.g., buthylsepharose, phenylsepharose, etc.), gel filtration with a molecular sieve,
15 affinity chromatography, chromatofocusing, electrophoresis (e.g., isoelectric focusing electrophoresis, etc.).

When the polypeptide of the present invention has been expressed and formed insoluble bodies within cells,
20 the cells are harvested, pulverized, and centrifuged. From the resulting precipitate fraction, the polypeptide of the present invention is collected using a commonly used method. The insoluble polypeptide is solubilized using a polypeptide denaturant. The resulting solubilized solution is diluted
25 or dialyzed into a denaturant-free solution or a dilute solution, where the concentration of the polypeptide denaturant is too low to denature the polypeptide. The polypeptide of the present invention is allowed to form a normal three-dimensional structure, and the purified
30 specimen is obtained by isolation and purification as described above.

Purification can be carried out in accordance with

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a commonly used protein purification method (J. Evan. Sadler et al.: Methods in Enzymology, 83, 458). Alternatively, the polypeptide of the present invention can be fused with other proteins to produce a fusion protein, and the fusion protein
5 can be purified using affinity chromatography using a substance having affinity to the fusion protein (Akio Yamakawa, Experimental Medicine, 13, 469-474 (1995)). For example, in accordance with a method described in Lowe et al., Proc. Natl. Acad. Sci., USA, 86, 8227-8231 (1989), Genes
10 Develop., 4, 1288(1990)), a fusion protein of the polypeptide of the present invention with protein A is produced, followed by purification with affinity chromatography using immunoglobulin G.

15 The polypeptide of the present invention can be purified with affinity chromatography using antibodies which bind to the polypeptide. The polypeptide of the present invention can be produced using an *in vitro* transcription/translation system in accordance with a known
20 method (J. Biomolecular NMR, 6, 129-134; Science, 242, 1162-1164; J. Biochem., 110, 166-168 (1991)).

Based on the amino acid information of a polypeptide as obtained above, the polypeptide can also be produced by
25 a chemical synthesis method, such as the Fmoc method (fluorenylmethyloxycarbonyl method), the tBoc method (t-butyloxycarbonyl method), or the like. The peptide can be chemically synthesized using a peptide synthesizer (manufactured by Advanced ChemTech, Applied Biosystems,
30 Pharmacia Biotech, Protein Technology instrument, Synthecell-Vega, PerSeptive, Shimazu, or the like).

(Substrate/plate/chip/array)

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As used herein, the term "plate" refers to a planar support onto which a molecule, such as an antibody or the like, may be fixed. In the present invention, a plate preferably comprises a glass substrate (base material), which has one side provided with a thin film made of a plastic, gold, silver or aluminum.

As used herein, the term "substrate" refers to a material (preferably solid material) with which a chip or array of the present invention is constructed. Therefore, a substrate is encompassed by the concept of a plate. Examples of materials for substrates include any solid materials to which a biological molecule used in the present invention is fixed via a covalent or noncovalent bond or which may be adapted to have such a property.

Examples of materials for plates and substrates include, but are not limited to, any material capable of forming solid surfaces, such as glass, silica, silicon, ceramics, silicon dioxide, plastics, metals (including alloys), naturally-occurring and synthetic polymers (e.g., polystyrene, cellulose, chitosan, dextran, and nylon), and the like. A substrate may be formed of a plurality of layers made of different materials. Examples of materials for plates and substrates include, but are not limited to, organic insulating materials, such as glass, quartz glass, alumina, sapphire, forsterite, silicon carbide, silicon oxide, silicon nitride, and the like. Examples of materials for plates and substrates also include, but are not limited to, organic materials, such as polyethylene, ethylene, polypropylene, polyisobutylene, polyethylene terephthalate, unsaturated polyester, fluorine-containing resin, polyvinyl chloride, polyvinylidene chloride, polyvinyl

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acetate, polyvinyl alcohol, polyvinyl acetal, acrylic resin, polyacrylonitrile, polystyrene, acetal resin, polycarbonate, polyamide, phenol resin, urea resin, epoxy resin, melamine resin, styrene-acrylonitrile copolymer, acrylonitrile-butadiene-styrene copolymer, silicone resin, polyphenylene oxide, polysulfone, and the like. A material preferable for a substrate varies depending on various parameters, such as measuring devices and the like, and can be selected as appropriate from the above-described various materials by those skilled in the art. For transfection arrays, glass slide is preferably. Preferably, the base material may be coated.

As used herein, the term "coating" in relation to a solid phase support or substrate refers to an act of forming a film of a material on a surface of the solid phase support or substrate, and also refers to a film itself. Coating is performed for various purposes, such as, for example, improvement in the quality of a solid phase support and substrate (e.g., elongation of life span, improvement in resistance to hostile environment, such as resistance to acids, etc.), an improvement in affinity to a substance integrated with a solid phase support or substrate, and the like. Such a substance used for coating is herein referred to as a "coating agent". Various materials may be used for such coating, including, without limitation, biological substances (e.g., DNA, RNA, protein, lipid, etc.), polymers (e.g., poly-L-lysine, MAS (available from Matsunami Glass, Kishiwada, Japan), and hydrophobic fluorine resin), silane (APS (e.g., γ -aminopropyl silane, etc.)), metals (e.g., gold, etc.), in addition to the above-described solid phase support and substrate. The selection of such materials is within the technical scope of those skilled in the art and thus

can be performed using techniques well known in the art. In one preferred embodiment, such a coating may be advantageously made of poly-L-lysine, silane (e.g., epoxy silane or mercaptosilane, APS (γ -aminopropyl silane), etc.),
5 MAS, hydrophobic fluorine resin, a metal (e.g., gold, etc.). Such a material may be preferably a substance suitable for cells or objects containing cells (e.g., organisms, organs, etc.).

10 As used herein, the terms "chip" or "microchip" are used interchangeably to refer to a micro integrated circuit which has versatile functions and constitutes a portion of a system. Examples of a chip include, but are not limited to, DNA chips, protein chips, and the like.

15 As used herein, the terms "array" and "bioassay" are used interchangeably to refer to a substrate (e.g., a chip, etc.) which has a pattern of a composition containing at least one (e.g., 1000 or more, etc.) target substances (e.g.,
20 DNA, proteins, transfection mixtures, etc.), which are arrayed. Among arrays, patterned substrates having a small size (e.g., 10x10 mm, etc.) is particularly referred to as microarrays. The terms "microarray" and "array" are used interchangeably. Therefore, a patterned substrate having
25 a larger size than that which is described above may be referred to as a microarray. For example, an array comprises a set of desired transfection mixtures fixed to a solid phase surface or a film thereof. An array preferably comprises at least 10^2 antibodies of the same or different types, more
30 preferably at least 10^3 , even more preferably at least 10^4 , and still even more preferably at least 10^5 . These antibodies are placed on a surface of up to 125x80 mm, more preferably 10x10 mm. An array includes, but is not limited to, a 96-well

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microtiter plate, a 384-well microtiter plate, a microtiter plate the size of a glass slide, and the like. A composition to be fixed may contain one or a plurality of types of target substances. Such a number of target substance types may be
5 in the range of from one to the number of spots, including, without limitation, about 10, about 100, about 500, and about 1,000.

As described above, any number of target substances
10 (e.g., proteins, such as antibodies) may be provided on a solid phase surface or film, typically including no more than 10^8 biological molecules per substrate, in another embodiment no more than 10^7 biological molecules, no more than 10^6 biological molecules, no more than 10^5 biological
15 molecules, no more than 10^4 biological molecules, no more than 10^3 biological molecules, or no more than 10^2 biological molecules. A composition containing more than 10^8 biological molecule target substances may be provided on a substrate. In these cases, the size of a substrate is preferably small.
20 Particularly, the size of a spot of a composition containing target substances (e.g., proteins such as antibodies) may be as small as the size of a single biological molecule (e.g., 1 to 2 nm order). In some cases, the minimum area of a substrate may be determined based on the number of biological
25 molecules on a substrate. A composition containing target substances, which are intended to be introduced into cells, are herein typically arrayed on and fixed via covalent bonds or physical interaction to a substrate in the form of spots having a size of 0.01 mm to 10 mm.

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"Spots" of biological molecules may be provided on an array. As used herein, the term "spot" refers to a certain set of compositions containing target substances. As used

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herein, the term "spotting" refers to an act of preparing a spot of a composition containing a certain target substance on a substrate or plate. Spotting may be performed by any method, for example, pipetting or the like, or alternatively, using an automatic device. These methods are well known in the art.

As used herein, the term "address" refers to a unique position on a substrate, which may be distinguished from other unique positions. Addresses are appropriately associated with spots. Addresses can have any distinguishable shape such that substances at each address may be distinguished from substances at other addresses (e.g., optically). A shape defining an address may be, for example, without limitation, a circle, an ellipse, a square, a rectangle, or an irregular shape. Therefore, the term "address" is used to indicate an abstract concept, while the term "spot" is used to indicate a specific concept. Unless it is necessary to distinguish them from each other, the terms "address" and "spot" may be herein used interchangeably.

The size of each address particularly depends on the size of the substrate, the number of addresses on the substrate, the amount of a composition containing target substances and/or available reagents, the size of microparticles, and the level of resolution required for any method used for the array. The size of each address may be, for example, in the range of from 1-2 nm to several centimeters, though the address may have any size suited to an array.

The spatial arrangement and shape which define an address are designed so that the microarray is suited to

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a particular application. Addresses may be densely arranged or sparsely distributed, or subgrouped into a desired pattern appropriate for a particular type of material to be analyzed.

5 Microarrays are widely reviewed in, for example, "Genomu Kino Kenkyu Purotokoru [Genomic Function Research Protocol] (Jikken Igaku Bessatsu [Special Issue of Experimental Medicine], Posuto Genomu Jidai no Jikken Koza 1 [Lecture 1 on Experimentation in Post-genome Era), "Genomu
10 Ikagaku to korekarano Genomu Iryo [Genome Medical Science and Futuristic Genome Therapy (Jikken Igaku Zokan [Special Issue of Experimental Medicine]), and the like.

 A vast amount of data can be obtained from a microarray.
15 Therefore, data analyzsis software is important for administration of correspondence between clones and spots, data analysis, and the like. Such software may be attached to various detection systems (e.g., Ermolaeva O. et al., (1998) Nat. Genet., 20: 19-23). The format of database
20 includes, for example, GATC (genetic analysis technology consortium) proposed by Affymetrix.

 Micromachining for arrays is described in, for example, Campbell, S.A. (1996), "The Science and Engineering
25 of Microelectronic Fabrication", Oxford University Press; Zaut, P.V. (1996), "Micromicroarray Fabrication: a Practical Guide to Semiconductor Processing", Semiconductor Services; Madou, M.J. (1997), "Fundamentals of Microfabrication", CRC1
5 Press; Rai-Choudhury, P. (1997), "Handbook of
30 Microlithography, Micromachining, & Microfabrication: Microlithography"; and the like, portions related thereto of which are herein incorporated by reference.

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(Cells)

The term "cell" is herein used in its broadest sense in the art, referring to a structural unit of tissue of a multicellular organism, which is capable of self replicating, has genetic information and a mechanism for expressing it, and is surrounded by a membrane structure which isolates the living body from the outside. Cells used herein may be either naturally-occurring cells or artificially modified cells (e.g., fusion cells, genetically modified cells, etc.). Examples of cell sources include, but are not limited to, a single-cell culture; the embryo, blood, or body tissue of normally-grown transgenic animal; a cell mixture of cells derived from normally-grown cell lines; and the like.

Cells used herein may be derived from any organism (e.g., any unicellular organisms (e.g., bacteria and yeast) or any multicellular organisms (e.g., animals (e.g., vertebrates and invertebrates), plants (e.g., monocotyledons and dicotyledons, etc.)). For example, cells used herein are derived from a vertebrate (e.g., Myxiniiformes, Petronyzoniformes, Chondrichthyes, Osteichthyes, amphibian, reptilian, avian, mammalian, etc.), more preferably mammalian (e.g., monotremata, marsupialia, edentate, dermoptera, chiroptera, carnivore, insectivore, proboscidea, perissodactyla, artiodactyla, tubulidentata, pholidota, sirenia, cetacean, primates, rodentia, lagomorpha, etc.). In one embodiment, cells derived from Primates (e.g., chimpanzee, Japanese monkey, human) are used. Particularly, without limitation, cells derived from a human are used.

As used herein, the term "stem cell" refers to a cell capable of self replication and pluripotency. Typically,

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stem cells can regenerate an injured tissue. Stem cells used herein may be, but are not limited to, embryonic stem (ES) cells or tissue stem cells (also called tissular stem cell, tissue-specific stem cell, or somatic stem cell). A stem cell may be an artificially produced cell (e.g., fusion cells, reprogrammed cells, or the like used herein) as long as it can have the above-described abilities. Embryonic stem cells are pluripotent stem cells derived from early embryos. An embryonic stem cell was first established in 1981, which has been applied to production of knockout mice since 1989. In 1998, a human embryonic stem cell was established, which is currently becoming available for regenerative medicine. Tissue stem cells have a relatively limited level of differentiation unlike embryonic stem cells. Tissue stem cells are present in tissues and have an undifferentiated intracellular structure. Tissue stem cells have a higher nucleus/cytoplasm ratio and have few intracellular organelles. Most tissue stem cells have pluripotency, a long cell cycle, and proliferative ability beyond the life of the individual. As used herein, stem cells may be preferably embryonic stem cells, though tissue stem cells may also be employed depending on the circumstance.

Tissue stem cells are separated into categories of sites from which the cells are derived, such as the dermal system, the digestive system, the bone marrow system, the nervous system, and the like. Tissue stem cells in the dermal system include epidermal stem cells, hair follicle stem cells, and the like. Tissue stem cells in the digestive system include pancreatic (common) stem cells, liver stem cells, and the like. Tissue stem cells in the bone marrow system include hematopoietic stem cells, mesenchymal stem cells, and the like. Tissue stem cells in the nervous system include

neural stem cells, retinal stem cells, and the like.

As used herein, the term "somatic cell" refers to any cell other than a germ cell, such as an egg, a sperm, or the like, which does not transfer its DNA to the next generation. Typically, somatic cells have limited or no pluripotency. Somatic cells used herein may be naturally-occurring or genetically modified as long as they can achieve the intended treatment.

10

The origin of a stem cell is categorized into the ectoderm, endoderm, or mesoderm. Stem cells of ectodermal origin are mostly present in the brain, including neural stem cells. Stem cells of endodermal origin are mostly present in bone marrow, including blood vessel stem cells, hematopoietic stem cells, mesenchymal stem cells, and the like. Stem cells of mesoderm origin are mostly present in organs, including liver stem cells, pancreas stem cells, and the like. Somatic cells may be herein derived from any germ layer. Preferably, somatic cells, such as lymphocytes, spleen cells or testis-derived cells, may be used.

20

As used herein, the term "isolated" means that naturally accompanying material is at least reduced, or preferably substantially completely eliminated, in normal circumstances. Therefore, the term "isolated cell" refers to a cell substantially free from other accompanying substances (e.g., other cells, proteins, nucleic acids, etc.) in natural circumstances. The term "isolated" in relation to nucleic acids or polypeptides means that, for example, the nucleic acids or the polypeptides are substantially free from cellular substances or culture media when they are produced by recombinant DNA techniques; or precursory

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chemical substances or other chemical substances when they are chemically synthesized. Isolated nucleic acids are preferably free from sequences naturally flanking the nucleic acid within an organism from which the nucleic acid is derived (i.e., sequences positioned at the 5' terminus and the 3' terminus of the nucleic acid).

As used herein, the term "established" in relation to cells refers to a state of a cell in which a particular property (pluripotency) of the cell is maintained and the cell undergoes stable proliferation under culture conditions. Therefore, established stem cells maintain pluripotency.

As used herein, the term "differentiated cell" refers to a cell having a specialized function and form (e.g., muscle cells, neurons, etc.). Unlike stem cells, differentiated cells have no or little pluripotency. Examples of differentiated cells include epidermic cells, pancreatic parenchymal cells, pancreatic duct cells, hepatic cells, blood cells, cardiac muscle cells, skeletal muscle cells, osteoblasts, skeletal myoblasts, neurons, vascular endothelial cells, pigment cells, smooth muscle cells, fat cells, bone cells, cartilage cells, and the like.

(Medicaments and cosmetics, and therapy and prevention using the same)

In another aspect, the present invention relates to medicaments (e.g., medicaments (vaccine, etc.), health foods, medicaments comprising a protein or lipid having reduced antigenicity, etc.), cosmetics, agricultural chemicals, foods, and the like, for introducing an effective ingredient into cells. Such medicaments and cosmetics may further comprise a pharmaceutically acceptable carrier. Such a

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pharmaceutically acceptable carrier contained in a medicament of the present invention includes any known substances.

5 Examples of a pharmaceutical acceptable carrier or a suitable formulation material include, but are not limited to, antioxidants, preservatives, colorants, flavoring agents, diluents, emulsifiers, suspending agents, solvents, fillers, bulky agents, buffers, delivery vehicles, and/or
10 pharmaceutical adjuvants. Representatively, a medicament of the present invention is administered in the form of a composition comprising a compound, or a variant or derivative thereof, with at least one physiologically acceptable carrier, excipient or diluent. For example, an appropriate vehicle
15 may be injection solution, physiological solution, or artificial cerebrospinal fluid, which can be supplemented with other substances which are commonly used for compositions for parenteral delivery.

20 Acceptable carriers, excipients or stabilizers used herein preferably are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and preferably include phosphate, citrate, or other organic acids; ascorbic acid, α -tocopherol; low molecular weight
25 polypeptides; proteins (e.g., serum albumin, gelatin, or immunoglobulins); hydrophilic polymers (e.g., polyvinylpyrrolidone); amino acids (e.g., glycine, glutamine, asparagine, arginine or lysine); monosaccharides, disaccharides, and other carbohydrates (glucose, mannose, or dextrans);
30 chelating agents (e.g., EDTA); sugar alcohols (e.g., mannitol or sorbitol); salt-forming counterions (e.g., sodium); and/or nonionic surfactants (e.g., Tween, pluronics or polyethylene glycol (PEG)).

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Examples of appropriate carriers include neutral buffered saline or saline mixed with serum albumin. Preferably, the product is formulated as a lyophilizate using appropriate excipients (e.g., sucrose). Other standard carriers, diluents, and excipients may be included as desired. Other exemplary compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor.

The medicament of the present invention may be administered orally or parenterally. Alternatively, the medicament of the present invention may be administered intravenously or subcutaneously. When systemically administered, the medicament for use in the present invention may be in the form of a pyrogen-free, pharmaceutically acceptable aqueous solution. The preparation of such pharmaceutically acceptable compositions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art. Administration methods may be herein oral, parenteral administration (e.g., intravenous, intramuscular, subcutaneous, intradermal, to mucosa, intrarectal, vaginal, topical to an affected site, to the skin, etc.). A prescription for such administration may be provided in any formulation form. Such a formulation form includes liquid formulations, injections, sustained preparations, and the like.

The medicament of the present invention may be prepared for storage by mixing a sugar chain composition having the desired degree of purity with optional physiologically acceptable carriers, excipients, or

stabilizers (Japanese Pharmacopeia ver. 14, or a supplement thereto or the latest version; Remington's Pharmaceutical Sciences, 18th Edition, A. R. Gennaro, ed., Mack Publishing Company, 1990; and the like), in the form of lyophilized cake or aqueous solutions.

The amount of the composition of the present invention used in the treatment method of the present invention can be easily determined by those skilled in the art with reference to the purpose of use, a target disease (type, severity, and the like), the patient's age, weight, sex, and case history, the form or type of the cell, and the like. The frequency of the treatment method of the present invention applied to a subject (or patient) is also determined by those skilled in the art with respect to the purpose of use, target disease (type, severity, and the like), the patient's age, weight, sex, and case history, the progression of the therapy, and the like. Examples of the frequency include once per day to several months (e.g., once per week to once per month). Preferably, administration is performed once per week to month with reference to the progression.

When the present invention is used for other applications, such as cosmetics, food, agricultural chemicals, and the like, it may be prepared in accordance with limitations defined by the authority.

(Description of preferred embodiments)

Hereinafter, the present invention will be described by way of embodiments. Embodiments described below are provided only for illustrative purposes. Accordingly, the scope of the present invention is not limited by the embodiments except as by the appended claims.

In one aspect, the present invention provides a composition for increasing the efficiency of introducing a target substance into a cell. The composition of the present invention comprises (a) an actin acting substance. The above-described object of the present invention was achieved by unexpectedly finding that the introduction of a substance (e.g., DNA, RNA, polypeptides, sugar chains or a composite substance thereof, etc.), which is not substantially introduced under normal conditions, is promoted by the action of an actin acting substance (representatively, an extracellular matrix protein). Particularly, it was found that such an actin acting substance has a significant effect of promoting introduction efficiency in genetic manipulation using DNA, such as transfection. Such a finding has not been conventionally known or expected. Attention should be focused onto the present invention which will be a significant breakthrough in gene research.

In a preferred embodiment, an actin acting substance used in the composition of the present invention may be an extracellular matrix protein or a variant or fragment thereof. In the present invention, it was found that an extracellular matrix protein or a variant or fragment thereof unexpectedly acts on actin. Therefore, attention should be focused onto an effect of increasing the efficiency of introducing a substance into cells due to an extracellular matrix protein according to the present invention.

Therefore, in another aspect, the present invention provides a composition for increasing the efficiency of introducing a target substance into a cell, which comprises an extracellular matrix protein or a variant or fragment

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thereof.

Examples of preferable actin acting substances contained in the composition of the present invention include, but are not limited to, fibronectin, pronectin F, pronectin L, pronectin Plus, laminin, vitronectin, or a variant or fragment thereof.

In a preferred embodiment, an actin acting substance contained in the composition of the present invention, comprises:

(a-1) a protein molecule having at least a Fn1 domain, or a variant thereof;

(a-2) a protein molecule having an amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11, or a variant or fragment thereof;

(b) a polypeptide having the amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11 having at least one mutation selected from the group consisting of at least one amino acid substitution, addition, and deletion, and having a biological activity;

(c) a polypeptide encoded by a splice or allelic mutant of a base sequence set forth in SEQ ID NO.: 1, 3, 5, 7 or 9;

(d) a polypeptide which is a species homolog of the amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11; or

(e) a polypeptide having an amino acid sequence having at least 70% identity to any one of the polypeptides (a-1) to (d), and having a biological activity.

In a preferred embodiment, the number of substitutions, additions, and deletions in (b) is preferably

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limited to, for example, 50 or less, 40 or less, 30 or less, 20 or less, 15 or less, 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In a certain particular embodiment, the number
5 of substitutions, additions, and deletions may be one or several. A smaller number of substitutions, additions, and deletions are preferable. However, a larger number of substitutions, additions, and deletions are possible as long as a biological activity is retained (preferably, an activity
10 which is similar to or the same as that of an actin acting substance).

In another preferred embodiment, the above-described allelic mutant may preferably have at least
15 90% homology to the nucleic acid sequence set forth in SEQ ID NO.: 1, 3, 5, 7 or 9. In the same line or the like, for example, such an allelic mutant may preferably have at least 99% homology. In another preferred embodiment, the allelic mutant of (c) may preferably have at least about 90% homology
20 to the amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11. Preferably, the allelic mutant of (c) may have at least about 99% homology to the amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11.

25 When a gene sequence database is available for the above-described species homolog, the species homolog can be identified by searching the database using the whole or a part of the gene sequence of the extracellular matrix protein of the present invention (e.g., fibronectin, vitronectin,
30 laminin, etc.) as a query sequence. Alternatively, the species homolog can be identified by screening gene libraries of the species using the whole or a part of the gene of the extracellular matrix protein of the present invention (e.g.,

fibronectin, vitronectin, laminin, etc.) as a probe or a primer. Such identifying methods are well known in the art and described in documents mentioned herein. The species homolog may preferably have at least about 30% homology to the nucleic acid sequence set forth in SEQ ID NO.: 1, 3, 5, 7 or 9, for example. The species homolog may preferably have at least about 50% homology to the nucleic acid sequence set forth in SEQ ID NO.: 1, 3, 5, 7 or 9. In another preferred embodiment, the species homolog may preferably have at least about 30% homology to the amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11. The species homolog may preferably have at least about 50% homology to the amino acid sequence set forth in SEQ ID NO.: 2, 4, 6, 8, 10 or 11.

15

In a preferred embodiment, the identity to any one of the polypeptides (a-1) to (d) may be at least about 80%, more preferably at least about 90%, even more preferably at least about 98%, and most preferably at least about 99%.

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In a more preferred embodiment, the nucleic acid sequence or amino acid sequence may be a sequence related to SEQ ID NO.: 1, 2 or 11 (fibronectin sequence). Therefore, the description "homology thereof" may be replaced with SEQ ID NO.: 1, 2 or 11 in a more preferred embodiment.

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In one embodiment, the actin acting substance of the present invention may comprise a Fn1 domain of amino acids 21 to 577 of SEQ ID NO.: 11.

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In another preferred embodiment, the actin acting substance may be fibronectin or a variant or fragment thereof, and more preferably fibronectin.

The concentration of the actin acting substance can be easily determined by those skilled in the art with reference to the present specification. For example, such a concentration may be at least about 0.1 $\mu\text{g}/\mu\text{L}$, preferably about 0.2 $\mu\text{g}/\mu\text{L}$, and more preferably 0.5 $\mu\text{g}/\mu\text{L}$. In one embodiment, the introduction efficiency reaches a plateau in the case of a concentration of about 0.5 $\mu\text{g}/\mu\text{L}$ or more. A preferable concentration range may be from about 0.5 $\mu\text{g}/\mu\text{L}$ to 2.0 $\mu\text{g}/\mu\text{L}$.

In another aspect, the present invention relates to a composition for increasing the efficiency of introducing a target substance into a cell, wherein the composition comprises an adhesion agent. Fibronectin has been known as an adhesion agent. However, it was not known that such an adhesion agent can be used to increase the efficiency of introducing a target substance into a cell (e.g., transfection, etc.). Therefore, the present invention can be considered to be attributed to the unexpected effect of adhesion agents. Such adhesion agents are described in detail above. Therefore, in the following various embodiments, such adhesion agents can be used instead of actin acting substances.

In an embodiment in which gene introduction is intended, the composition of the present invention may preferably comprise a gene introduction reagent. This is because such a gene introduction reagent synergistically exhibits the effect of increasing the efficiency of introduction of the present invention.

In a preferred embodiment, such a gene introduction

reagent includes, but is not limited to, at least one substance selected from the group consisting of cationic polymers, cationic lipids, and calcium phosphate. More preferably, examples of gene introduction reagents include, but are not limited to, Effectene, TransFastTM, TfxTM-20, SuperFect, PolyFect, LipofectAMINE 2000, JetPEI, ExGen 500, and the like.

In another embodiment, the composition of the present invention may further comprise a particle. This is because use of such a particle can lead to an increase in the efficiency of introducing a substance into a cell, particularly a target cell. Preferable examples of such a particle include, but are not limited to, metal colloids, such as gold colloid, and the like.

In another preferred embodiment, the composition of present invention may further comprise a salt. Though not wishing to be bound by any theory, use of such a salt enhances the fixing effect when a solid phase support is used. Alternatively, it is considered that the three-dimensional structure of a target substance can be retained in a more appropriate form.

Any inorganic or organic salt may be used as the above-described salt. Use of a mixture of a plurality of salts is more preferable than use of a single salt. Examples of such a mixture of a plurality of salts include, but are not limited to, salts contained in buffers, salts contained in media, and the like.

In another aspect, the present invention provides a kit for increasing the efficiency of introducing a gene.

The kit comprises: (a) a composition comprising an actin acting substance; and (b) a gene introduction reagent. Such an actin acting substance may be selected and used as described in detail above for the composition of the present invention for increasing the efficiency of introducing a target substance into a cell. An appropriate form of the actin acting substance can be selected by those skilled in the art based on the present specification. When the present invention is provided in the form of such a kit, the kit may comprise instructions. The instructions may be prepared in accordance with a format defined by an authority of a country in which the present invention is practiced, explicitly describing that the instructions are approved by the authority. The present invention is not limited to this. The instructions are typically provided in the form of a manual and in paper media. The instructions are not so limited and may be provided in the form of electronic media (e.g., web sites, electronic mails, and the like provided on the Internet). Such an actin acting substance may be selected and used as described in detail above for the composition of the present invention for increasing the efficiency of introducing a target substance into a cell. Therefore, preferably, the actin acting substance may be an extracellular matrix protein (e.g., fibronectin, vitronectin, laminin, etc.) or a variant thereof. More preferably, fibronectin or a variant or fragment thereof may be used.

In another aspect, the present invention provides a composition for introducing a target substance into a cell. The present invention was completed by unexpectedly finding that the introduction of a substance (e.g., DNA, RNA, polypeptides, sugar chains or a composite substance thereof,

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etc.), which is not substantially introduced under normal conditions, is promoted by the action of an actin acting substance (representatively, an extracellular matrix protein). In this case, the present invention is provided
5 in the form of a composition comprising a target substance and an actin acting substance. Such an actin acting substance may be selected and used as described in detail above for the composition of the present invention for increasing the efficiency of introducing a target substance into a cell.
10 Therefore, preferably, the actin acting substance may be an extracellular matrix protein (e.g., fibronectin, vitronectin, laminin, etc.) or a variant thereof. More preferably, fibronectin or a variant or fragment thereof may be used.

15 Examples of a target substance contained in the composition of the present invention for introducing the target substance into a cell include, but are not limited to, DNA, RNA, polypeptides, sugars, and complexes thereof,
20 and the like. In a particular preferred embodiment, DNA may be selected as a target substance. Such DNA may preferably encode a gene of interest when gene expression is intended. Therefore, in an embodiment in which transfection is intended, a target substance may include DNA encoding a gene sequence
25 to be transfected. In another preferred embodiment, RNA is selected as a target substance. Such RNA may preferably encode a gene of interest when gene expression is intended. In this case, RNA encoding a gene sequence may be preferably used along with a gene introduction agent suitable for RNA.

30 In an embodiment in which gene introduction is intended, the composition of the present invention for introducing a target substance into a cell may further

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comprise a gene introduction reagent. Though not wishing to be bound by any theory, in one embodiment, it is considered that such a gene introduction reagent and an actin acting substance found in the present invention function in cooperation with each other, thereby achieving a higher efficiency of introducing a gene into a cell than that of conventional techniques.

In a preferred embodiment, examples of such a gene introduction reagent contained in the composition of the present invention include, but are not limited to, cationic polymers, cationic lipids, polyamine-based reagents, polyimine-based reagents, calcium phosphate, and the like.

In a preferred embodiment, the composition of the present invention for introducing a target substance into a cell may be a liquid phase. In the case of a liquid phase, the present invention is useful as, for example, a liquid phase transfection system.

In another preferred embodiment, the composition of the present invention for introducing a target substance into a cell may be a solid phase. In the case of a solid phase, the present invention is useful as, for example, a solid phase transfection system. Preferable examples of such a solid phase transfection system include, but are not limited to, microtiter plate-based transfection systems, array (or chip) -based transfection systems, and the like. For the introduction of a polypeptide, either a liquid phase or a solid phase may be useful.

In another aspect, the present invention provides a device for introducing a target substance into a cell.

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In the device, a composition comprising A) the target substance and B) an actin acting substance is fixed onto a solid phase support. The device of the present invention was completed by unexpectedly finding that the introduction of a substance (e.g., DNA, RNA, polypeptides, sugar chains or a composite substance thereof, etc.), which is not substantially introduced under normal conditions, is promoted by the action of an actin acting substance (representatively, an extracellular matrix protein). In this case, a composition comprising a target substance and an actin acting substance is fixed onto a solid phase support. Such an actin acting substance may be selected and used as described in detail above for the composition of the present invention for increasing the efficiency of introducing a target substance into a cell. Therefore, preferably, the actin acting substance may be an extracellular matrix protein (e.g., fibronectin, vitronectin, laminin, etc.) or a variant thereof. More preferably, fibronectin or a variant or fragment thereof may be used.

Examples of a target substance contained in the device of the present invention for introducing the target substance into a cell include, but are not limited to, DNA, RNA, polypeptides, sugars, and complexes thereof, and the like. In a particular preferred embodiment, DNA may be selected as a target substance. Such DNA may preferably encode a gene of interest when gene expression is intended. Therefore, in an embodiment in which transfection is intended, a target substance may include DNA encoding a gene sequence to be transfected.

In an embodiment in which gene introduction is intended, the device of the present invention may further

comprise a gene introduction reagent. Though not wishing to be bound by any theory, in one embodiment, it is considered that such a gene introduction reagent and an actin acting substance found in the present invention function in cooperation with each other, thereby achieving a higher efficiency of introducing a gene into a cell than that of conventional techniques.

In a preferred embodiment, a solid phase support used in the device of the present invention may be selected from the group consisting of plates, microwell plates, chips, slide glasses, films, beads, and metals.

In a particular embodiment, when the device of the present invention uses a chip as a solid phase support, the device may be called an array. In such an array, biological molecules (e.g., DNA, proteins, etc.) to be introduced are typically arranged or patterned on a substrate. Such an array used for transfection is also herein called a transfection array. In the present invention, it was revealed that transfection takes place for stem cells, which cannot be achieved by conventional systems. Therefore, the composition, device and method of the present invention which use an actin acting substance can be used to provide a transfection array capable of transfection of any cell. This is an unexpected effect which cannot be conventionally achieved.

A solid phase support used in the device of the present invention may be preferably coated. Coating improves the quality of a solid phase support and substrate (e.g., elongation of life span, improvement in resistance to hostile environment, such as resistance to acids, etc.), affinity

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to a substance integrated with a solid phase support or substrate, and the like. In a preferred embodiment, such coating is obtained with a coating agent, such as poly-L-lysine, silane (e.g., APS (γ -aminopropyl silane)), MAS, hydrophobic fluorine resin, silane (e.g., epoxy silane or mercaptosilane), a metal (e.g., gold, etc.), or the like. Preferably, a coating agent may be poly-L-lysine.

In another aspect, the present invention provides a method for increasing the efficiency of introducing a target substance into a cell. The present invention represents a first discovery and was completed by unexpectedly finding that the introduction of a substance (e.g., DNA, RNA, polypeptides, sugar chains or a composite substance thereof, etc.), which is not substantially introduced under normal conditions, is efficiently introduced into cells by presenting (preferably contacting) the target substance along with an actin acting substance to the cells. The method of the present invention comprises: A) providing the target substance; B) providing an actin acting substance; and further C) contacting the target substance and the actin acting substance to the cell. The target substance and the actin acting substance may be provided together or separately. Such an actin acting substance may be selected and used as described in detail above for the composition of the present invention for increasing the efficiency of introducing a target substance into a cell. Such selection may be made as appropriate by those skilled in the art based on the present specification. Therefore, preferably, the actin acting substance may be an extracellular matrix protein (e.g., fibronectin, vitronectin, laminin, etc.) or a variant thereof. More preferably, fibronectin or a variant or fragment thereof may be used.

Examples of a target substance contained in the method of the present invention include, but are not limited to, DNA, RNA, polypeptides, sugars, and complexes thereof, and the like. In a particular preferred embodiment, DNA may be selected as a target substance. Such DNA may preferably encode a gene of interest when gene expression is intended. Therefore, in an embodiment in which transfection is intended, a target substance may include DNA encoding a gene sequence to be transfected.

In an embodiment in which gene introduction is intended, the method of the present invention may further comprise a gene introduction reagent. Though not wishing to be bound by any theory, in one embodiment, it is considered that such a gene introduction reagent and an actin acting substance found in the present invention function in cooperation with each other, thereby achieving a higher efficiency of introducing a gene into a cell than that of conventional techniques. The gene introduction reagent and the target substance and/or the actin acting substance may be provided together or separately. Preferably, the target substance and the gene introduction reagent may be advantageously formed into a complex before providing the actin acting substance. Though not wishing to be bound by any theory, it is considered that introduction efficiency is increased by providing the target substance and the like in such an order.

In a preferred embodiment, examples of such a gene introduction reagent used in the method of the present invention include, but are not limited to, cationic polymers, cationic lipids, polyamine-based reagents, polyimine-based

reagents, calcium phosphate, and the like.

Any cell can be targeted in the present invention as long as the introduction of a target substance is intended. Examples of cells include, but are not limited to, stem cells, somatic cells, and the like. The present invention has a significant effect that a target substance can be introduced (e.g., transfected, etc.) into substantially all types of cells (e.g., stem cells, somatic cells, etc.). This effect can be said to be an unexpected effect which is not possessed by conventional methods. Preferably, target stem cells may include, without limitation, tissue stem cells and also embryonic stem cells. Though not wishing to be bound by any theory, among stem cells, it is considered that tissue stem cells have higher introduction efficiency than that of embryonic stem cells.

In a particular embodiment, a part or the whole of the method of the present invention for introducing a target substance into a cell may be performed in a liquid phase. In another particular embodiment, a part or the whole of the method of the present invention for introducing a target substance into a cell may be performed on a solid phase. Therefore, the method of the present invention for introducing a target substance into a cell may be performed using a combination of a liquid phase and a solid phase.

In another aspect, the present invention provides a method for increasing the efficiency of introducing a target substance into a cell using a solid phase support. The present invention represents a first discovery and was completed by unexpectedly finding that the introduction of a substance (e.g., DNA, RNA, polypeptides, sugar chains or

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a composite substance thereof, etc.), which is not substantially introduced under normal conditions, is efficiently introduced into cells by presenting (preferably contacting) the target substance along with an actin acting substance to the cells. The effect of increasing introduction efficiency of a target substance (particularly DNA, preferably DNA containing a sequence encoding a gene to be transfected) by using a solid phase support cannot be achieved, or at least expected, by conventional techniques. Thus, the present invention is a significant breakthrough in the art. The method of the present invention using a solid phase support comprises: I) fixing a composition comprising A) a target substance and B) an actin acting substance to a solid support; and II) contacting the cell to the composition on the solid support. Such an actin acting substance may be selected and used as described in detail above for the composition of the present invention for increasing the efficiency of introducing a target substance into a cell. Such selection may be made as appropriate by those skilled in the art based on the present specification. Preferably, the actin acting substance may be an extracellular matrix protein (e.g., fibronectin, vitronectin, laminin, etc.) or a variant thereof. More preferably, fibronectin or a variant or fragment thereof may be used.

Naked DNA may be used as a target substance. Preferably, DNA may be advantageously provided along with a control sequence (e.g., a promoter, etc.) using a vector (e.g., a plasmid, etc.). In such a case, preferably, DNA may be operably linked to be the control sequence.

Preferably, the method of the present invention may

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further comprise providing a gene introduction reagent, wherein the gene introduction reagent is contacted with the cell. Use of a gene introduction reagent is preferable because of a further improvement in introduction efficiency of the method of the present invention. It is well known in the art to provide a gene introduction reagent. For example, without limitation, a solution containing a gene introduction reagent dissolved therein is added to an experimentation system. Preferably, a gene introduction reagent and DNA (a target substance) are formed into a complex before providing an actin acting substance. Though not wishing to be bound by any theory, it was revealed that by providing the target substance and the like in such an order, the efficiency of introducing a target substance into a cell on a solid phase support is dramatically increased.

In one embodiment, the gene introduction reagent (e.g., cationic lipid)-target substance complex comprises a target substance (e.g., DNA in an expression vector) and a gene introduction reagent and is dissolved in an appropriate solvent, such as water or deionized water. The resultant solution is spotted onto a surface of a slide or the like, thereby producing a surface on which the gene introduction reagent-target substance complex is adhered to specific positions. Thereafter, an actin acting substance is added as appropriate. The spots of the gene introduction reagent-target substance complex are adhered to the slide, and are dried well so that the spots will remain adhered to the same position under the subsequent steps in the method. For example, a gene introduction reagent-target substance complex is spotted on a slide (e.g., a glass slide, etc.) or chip coated with poly-L-lysine (available from Sigma, Inc., etc.) manually or using a microarray producing machine.

Thereafter, the slide or chip is dried under reduced pressure at room temperature or a temperature higher than room temperature, thereby adhering the DNA spots onto the slide. The time required for drying well depends on several factors, such as the amount of a mixture provided on the surface, the temperature and humidity conditions, and the like. In the present invention, the actin acting substance may be preferably provided after adhesion of the complex.

The concentration of DNA in a mixture may be experimentally determined, but is generally in the range of from about 0.01 $\mu\text{g}/\mu\text{l}$ to about 0.2 $\mu\text{g}/\mu\text{l}$. In a particular embodiment, the range is from about 0.02 $\mu\text{g}/\mu\text{l}$ to about 0.10 $\mu\text{g}/\mu\text{l}$. Alternatively, the concentration of DNA in a gene introduction reagent-target substance complex is in the range of from about 0.01 $\mu\text{g}/\mu\text{l}$ to about 0.5 $\mu\text{g}/\mu\text{l}$, from about 0.01 $\mu\text{g}/\mu\text{l}$ to about 0.4 $\mu\text{g}/\mu\text{l}$, or from about 0.01 $\mu\text{g}/\mu\text{l}$ to about 0.3 $\mu\text{g}/\mu\text{l}$. Similarly, the concentration of another carrier polymer, such as an actin acting substance or a gene introduction reagent, may be experimentally determined for each application, but are generally in the range of from 0.01% to 0.5%. In a particular embodiment, the range is from about 0.05% to about 0.5%, from about 0.05% to about 0.2%, or from about 0.1% to about 0.2%. The final concentration of DNA (e.g., DNA in an actin acting substance) in an actin acting substance-target substance is generally in the range of from about 0.02 $\mu\text{g}/\mu\text{l}$ to about 0.1 $\mu\text{g}/\mu\text{l}$. In another embodiment, DNA may have a final concentration of about 0.05 $\mu\text{g}/\mu\text{l}$.

DNA used in the present invention may be provided in a vector of any type, such as a plasmid or a virus. A vector containing DNA of interest may be introduced into

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a cell, and thereafter, DNA may be expressed in the cell. For example, a CMV driven expression vector may be used. Commercially available plasmid vectors (e.g., pEGFP (Clontech) or pcDNA 3 (Invitrogen), etc.) or viral vectors may be used. In this embodiment, after the spots containing the gene introduction reagent-target substance complex is dried, the surface having the spots is coated with a transfection reagent based on an appropriate amount of lipid. The resultant product is maintained (incubated) under conditions suited for the formation of a complex of the DNA and the gene introduction reagent (e.g., a transfection reagent, such as a cationic lipid, etc.) in the spot. Preferably, an actin acting substance may be provided subsequently or simultaneously. In one embodiment, the resultant product is incubated at 25°C for about 20 minutes. Thereafter, the gene introduction reagent is removed. Thus, the surface having DNA (DNA in a complex of the DNA and the transfection reagent) is produced. Cells in appropriate culture medium are plated on the surface. The resultant product (the surface having the DNA and the plated cells) is maintained under conditions which allow the DNA to enter the plated cells.

In the present invention, a time of about 1 to 2 cell cycles is sufficient for transfection. The time required for transfection varies depending on the cell type and conditions. The time appropriate for a specific combination may be experimentally determined by those skilled in the art. After a sufficient time has passed, transfection efficiency, expression of encoded products, an influence on cells, and the like can be evaluated using known methods. For example, these parameters can be determined by detection of immunofluorescence, or enzymatic immunological cytology,

in situ hybridization, autoradiography, or other means for detecting an influence of DNA expression or DNA products or DNA itself on cells having the introduced DNA. When immunofluorescence is used for detection of expression of a protein encoded by DNA, an antibody which binds to a protein and is tagged with a fluorescent label (e.g., an antibody is applied to a slide under appropriate conditions which allow the antibody to bind to a protein) is used and a position (a spot or region on a surface) containing a protein is identified by detecting fluorescence. The presence of fluorescence indicates that transfection occurs at a position from which the fluorescence is emitted, i.e., the encoded protein is expressed. The presence of a signal detected on the slide by the above-described method indicates that transfection and expression of a coded product or introduction of DNA into the cell occur at a position from which the signal is detected. The identity of DNA provided at specific positions may be either known or unknown. Therefore, when expression occurs, the identity of an expressed protein may be either known or unknown. Such information may be preferably known. This is because such information can be correlated with conventional information.

All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein.

The preferred embodiments of the present invention have been heretofore described for a better understanding of the present invention. Hereinafter, the present invention will be described by way of examples. Examples described below are provided only for illustrative purposes. Accordingly, the scope of the present invention is not limited

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by the embodiments and examples specified herein except as by the appended claims.

EXAMPLES

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Hereinafter, the present invention will be described in greater detail by way of examples, though the present invention is not limited to the examples below. Reagents, supports, and the like were commercially available from Sigma (St. Louis, USA), Wako Pure Chemical Industries (Osaka, Japan), Matsunami Glass (Kishiwada, Japan) unless otherwise specified.

(Example 1: Preparation of actin acting substance mixture)

Formulations below were prepared in Example 1.

As candidates for an actin acting substance, various extracellular matrix proteins and variants or fragments thereof were prepared in Example 1 as listed below. Fibronectin and the like were commercially available. Fragments and variants were obtained by genetic engineering techniques:

- 1) fibronectin (SEQ ID NO.: 11);
- 2) fibronectin 29 kDa fragment;
- 3) fibronectin 43 kDa fragment;
- 4) fibronectin 72 kDa fragment;
- 5) fibronectin variant (SEQ ID NO.: 11, alanine at 152 was substituted with leucine);
- 6) pronectin F (Sanyo Chemical Industries, Kyoto, Japan);
- 7) pronectin L (Sanyo Chemical Industries);
- 8) pronectin Plus (Sanyo Chemical Industries);

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- 9) laminin (SEQ ID NO.: 6);
10) RGD peptide (tripeptide);
11) RGD-containing 30-kDa peptide;
12) 5 amino acids of laminin (SEQ ID NO.: 17); and
5 13) gelatin.

Plasmids were prepared as DNA for transfection. Plasmids, pEGFP-N1 and pDsRed2-N1 (both from BD Biosciences, Clontech, CA, USA) were used. In these plasmids, gene
10 expression was under the control of cytomegalovirus (CMV). The plasmid DNA was amplified in E. coli (XL1 blue, Stratgene, TX, USA) and the amplified plasmid DNA was used as a complex partner. The DNA was dissolved in distilled water free from DNase and RNase.

15

The following transfection reagents were used: Effectene Transfection Reagent (cat. no. 301425, Qiagen, CA), TransFast™ Transfection Reagent (E2431, Promega, WI), Tfx™-20 Reagent (E2391, Promega, WI), SuperFect Transfection
20 Reagent (301305, Qiagen, CA), PolyFect Transfection Reagent (301105, Qiagen, CA), LipofectAMINE 2000 Reagent (11668-019, Invitrogen corporation, CA), JetPEI (x4) conc. (101-30, Polyplus-transfection, France), and ExGen 500 (R0511, Fermentas Inc., MD). These transfection reagents were added
25 to the above-described DNA and actin acting substance in advance or complexes thereof with the DNA were produced in advance.

The thus-obtained solution was used in assays using
30 transfection arrays described below.

(Example 2: Improvement in transfection efficiency in liquid phase)

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In Example 2, an improvement in the transfection efficiency of solid phase was observed. The protocol used in Example 2 will be described below.

5

The protocol for liquid phase transfection is in accordance with instructions provided along with each of Effectene, LipofectAMINE 2000, JetPEI, or TransFast.

10

In Example 2, effects of the above-prepared actin acting substances were studied in the presence or absence thereof in liquid phase transfection.

15

An actin acting substance was preserved as a stock having a concentration of 10 $\mu\text{g}/\mu\text{L}$ in ddH_2O . All dilutions were made using PBS, ddH_2O , or Dulbecco's MEM. A series of dilutions, for example, 0.2 $\mu\text{g}/\mu\text{L}$, 0.27 $\mu\text{g}/\mu\text{L}$, 0.4 $\mu\text{g}/\mu\text{L}$, 0.53 $\mu\text{g}/\mu\text{L}$, 0.6 $\mu\text{g}/\mu\text{L}$, 0.8 $\mu\text{g}/\mu\text{L}$, 1.0 $\mu\text{g}/\mu\text{L}$, 1.07 $\mu\text{g}/\mu\text{L}$, 1.33 $\mu\text{g}/\mu\text{L}$, and the like, were formulated.

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As a result, it was revealed that these actin acting substances increased the efficiency of liquid phase transfection. Particularly, it was revealed that fibronectin had a significant effect of increasing the efficiency.

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(Example 3: Improvement in transfection efficiency in solid phase)

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In Example 3, an improvement in the transfection efficiency of solid phase was observed. The protocol used in Example 3 will be described below.

(Protocol)

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The final concentration of DNA was adjusted to 1 $\mu\text{g}/\mu\text{L}$. An actin acting substance was preserved as a stock having a concentration of 10 $\mu\text{g}/\mu\text{L}$ in ddH_2O . All dilutions were made using PBS, ddH_2O , or Dulbecco's MEM. A series of dilutions, for example, 0.2 $\mu\text{g}/\mu\text{L}$, 0.27 $\mu\text{g}/\mu\text{L}$, 0.4 $\mu\text{g}/\mu\text{L}$, 0.53 $\mu\text{g}/\mu\text{L}$, 0.6 $\mu\text{g}/\mu\text{L}$, 0.8 $\mu\text{g}/\mu\text{L}$, 1.0 $\mu\text{g}/\mu\text{L}$, 1.07 $\mu\text{g}/\mu\text{L}$, 1.33 $\mu\text{g}/\mu\text{L}$, and the like, were formulated.

Transfection reagents were used in accordance with instructions provided by each manufacturer.

Plasmid DNA was removed from a glycerol stock and amplified in 100 mL L-amp overnight. Qiaprep Miniprep or Qiagen Plasmid Purification Maxi was used to purify DNA in accordance with a standard protocol provided by the manufacturer.

In Example 3, the following 5 cells were used to confirm an effect: human mesenchymal stem cell (hMSCs, PT-2501, Cambrex BioScience Walkersville, Inc., MD); human embryonic renal cell (HEK293, RCB1637, RIKEN Cell Bank, JPN); NIH3T3-3 cell (RCB0150, RIKEN Cell Bank, JPN); HeLa cell (RCB0007, RIKEN Cell Bank, JPN); and HepG2 (RCB1648, RIKEN Cell Bank, JPN). These cells were cultured in DMEM/10% FCS containing L-glut and pen/strep.

(Dilution and DNA spots)

Transfection reagents and DNA were mixed to form a DNA-transfection reagent complex. The complex formation requires a certain period of time. Therefore, the mixture was spotted onto a solid phase support (e.g., a poly-L-lysine slide) using an arrayer. In Example 3, as a solid phase support, an APS slide, a MAS slide, and a uncoated slide

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were used as well as a poly-L-lysine slide. These slides are available from Matsunami Glass (Kishiwada, Japan) or the like.

5 For complex formation and spot fixation, the slides were dried overnight in a vacuum dryer. Drying was performed in the range of 2 hours to 1 week.

10 Although the actin acting substance might be used during the complex formation, it was also used immediately before spotting in Example 3.

(Formulation of mixed solution and application to solid phase supports)

15 300 μ L of DNA concentrated buffer (EC buffer) + 16 μ L of an enhancer were mixed in an Eppendorf tube. The mixture was mixed with a Vortex, followed by incubation for 5 minutes. 50 μ L of a transfection reagent (Effectene, etc.) was added to the mixture, followed by mixing by pipetting. To apply
20 a transfection reagent, an annular wax barrier was formed around the spots on the slide. 366 μ L of the mixture was added to the spot region surrounded by the wax, followed by incubation at room temperature for 10 to 20 minutes. Thereby, the fixation to the support was manually achieved.

25 (Distribution of cells)

Next, a protocol for adding cells will be described. Cells were distributed for transfection. The distribution was typically performed by reduced-pressure suction in a
30 hood. A slide was placed on a dish, and a solution containing cells was added to the dish for transfection. The cells were distributed as follows.

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The growing cells were distributed to a concentration of 10^7 cells/25 mL. The cells were plated on the slide in a 100x100x15 mm squared Petri dish or a 100 mm (radius) x 15 mm circular dish. Transfection was conducted for about 5 40 hours. This period of time corresponded to about 2 cell cycles. The slide was treated for immunofluorescence.

(Evaluation of gene introduction)

Gene introduction was evaluated by detection using, 10 for example, immunofluorescence, fluorescence microscope examination, laser scanning, radioactive labels, and sensitive films, or emulsion.

When an expressed protein to be visualized is a 15 fluorescent protein, such a protein can be observed with a fluorescence microscope and a photograph thereof can be taken. For large-sized expression arrays, slides may be scanned using a laser scanner for storage of data. If an expressed protein can be detected using fluorescence 20 antibodies, an immunofluorescence protocol can be successively performed. If detection is based on radioactivity, the slide may be adhered as described above, and autoradiography using film or emulsion can be performed to detect radioactivity.

25

(Laser scanning and Quantification of fluorescence intensity)

To quantify transfection efficiency, the present inventors use a DNA microarray scanner (GeneTAC UC4x4, 30 Genomic Solutions Inc., MI). Total fluorescence intensity (arbitrary unit) was measured, and thereafter, fluorescence intensity per unit surface area was calculated.

(Cross-sectional observation by confocal scanning microscope)

Cells were seeded on tissue culture dishes at a final concentration of 1×10^5 cells/well and cultured in appropriate medium (Human Mesenchymal Cell Basal Medium (MSCGM BulletKit PT-3001, Cambrex BioScience Walkersville, Inc., MD). After fixation of the cell layer with 4% paraformaldehyde solution, SYTO and Texas Red-X phalloidin (Molecular Probes Inc., OR, USA) was added to the cell layer for observation of nuclei and F-actin. The samples emitting light due to gene products and the stained samples were observed with a confocal laser microscope (LSM510: Carl Zeiss Co., Ltd., pin hole size=Ch1=123 μm , Ch2=108 μm , image interval = 0.4) to obtain cross sectional views.

15

(Results)

Figure 1 shows the results of experiments in which various actin acting substances and HEK293 cells were used where gelatin was used as a control.

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As can be seen from the results, whereas transfection was not very successful in a system using gelatin, transfection took place to a significant level in systems using fibronectin, pronectin (pronectin F, pronectin L, pronectin Plus) which is a variant of fibronectin, and laminin. Therefore, it was demonstrated that these molecules significantly increased transfection efficiency. Use of the RGD peptide alone exhibited substantially no effect.

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Figures 2 and 3 show transfection efficiency when fibronectin fragments were used. Figure 4 shows the summary of the results. 29 kDa and 72 kDa fragments exhibited a significant level of transfection activity, while a 43 kDa

fragment had activity but its level was low. Therefore, it was suggested that an amino acid sequence contained in the 29 kDa fragment played a role in an increase in transfection efficiency. Substantially no contamination was found in the case of the 29 kDa fragment, while contamination was observed in the case of the other two fragments (43 kDa and 72 kDa). Therefore, only the 29 kDa domain may be preferably used as an actin acting substance. When only the RGD peptide was used, the activity to increase transfection efficiency was not exhibited. The 29-kDa peptide exhibited activity. Such a system with additional 6 amino acids of laminin (higher molecular weight) exhibited transfection activity. Therefore, these peptide sequences may also play an important role in the activity to increase transfection efficiency, without limitation. In such a case, a molecular weight of at least 5 kDa, preferably at least 10 kDa, and more preferably at least 15 kDa may be required for an increase in transfection efficiency.

Next, Figure 5 shows the result of studies on transfection efficiency of cells. In Figure 5, HEK293 cells, HeLa cells, and 3T3 cells, which were conventionally transfectable, and HepG2 cells and mesenchymal stem cells (MSC) which were conventionally believed to be substantially impossible to transfect, were used to show an effect of the transfection method of the present invention. The vertical axis represents the intensity of GFP.

In Figure 5, the transfection method of the present invention using a solid phase support was compared with a conventional liquid phase transfection method. The conventional liquid phase transfection method was conducted in accordance with a protocol recommended by the kit

manufacturer.

As can be seen from Figure 5, transfection efficiency comparable to HeLa and 3T3 was achieved in HepG2 cells and mesenchymal stem cells (MSC) which were conventionally believed to be substantially impossible to transfect, as well as HEK293 cells, HeLa cells, and 3T3 cells, which were conventionally transfectable. Such an effect was not achieved by conventional transfection systems. The present invention was the first to provide a system which can increase transfection efficiency for substantially all cells and can provide practicable transfection to all cells. By using solid phase conditions, cross contamination was significantly reduced. Therefore, it was demonstrated that the present invention using a solid phase support is appropriate for production of an integrated bioarray.

Next, Figure 6 shows the results of transfection when various plates were used. As can be seen from the results of Figure 6, when coating was provided, contamination was reduced as compared with when coating was not provided and transfection efficiency was increased.

Next, Figure 7 shows the results of transfection where the concentration of fibronectin was 0, 0.27, 0.53, 0.8, 1.07, and 1.33 ($\mu\text{g}/\mu\text{L}$ for each). In Figure 7, slides coated with PLL (poly-L-lysine) and APS and uncoated slides were shown.

As can be seen from the results of Figure 7, transfection efficiency was increased with an increase in fibronectin concentration. Note that in the case of PLL coating and the absence of coating, the transfection

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efficiency reached a plateau at a fibronectin concentration of more than 0.53 $\mu\text{g}/\mu\text{L}$. In the case of APS, it was found that the effect was further increased at a fibronectin concentration of more than of 1.07 $\mu\text{g}/\mu\text{L}$.

5

Next, Figure 8 shows photographs indicating cell adhesion profiles in the presence or absence of fibronectin. Figure 9 shows cross-sectional photographs. It was revealed that the shapes of adherent cells were significantly different (Figure 8). The full extension of cells was found for the initial 3 hours of culture in the presence of fibronectin, while extension was limited in the absence of fibronectin (Figure 9). Considering the behavior of filaments (Figure 9) and the results of the time-lapse observation, it was considered that an actin acting substance, such as fibronectin, attached to a solid phase support had an influence on the shape and orientation of actin filaments, and the efficiency of introduction of a substance into a cell, such as transfection efficiency or the like, is increased. Specifically, actin filaments quickly change their location in the presence of fibronectin, and disappear from the cytoplasmic space under the nucleus as the cell extends. It is considered that actin depletion in the perinuclear space, which is induced by an actin acting substance, such as fibronectin, allows the transport of a target substance, such as DNA or the like, into cells or nuclei. Though not wishing to be bound by any theory, the reason is considered to be that the viscosity of cytoplasm is reduced and positively charged DNA particles are prevented from being trapped by negatively charged actin filaments. Additionally, it is considered that the surface area of the nucleus is significantly increased in the presence of fibronectin (Figure 10), possibly facilitating the transfer

30

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of a target substance, such as DNA or the like, into nuclei.

(Example 4: Application to bioarrays)

Next, larger-scale experiments were conducted to
5 determine whether or not the above-described effect was
demonstrated when arrays were used.

(Experimental protocols)

(Cell sources, culture media, and culture
10 conditions)

In this example, five different cell lines were used:
human mesenchymal stem cells (hMSCs, PT-2501, Cambrex
BioScience Walkersville, Inc., MD), human embryonic kidney
cell HEK293 (RCB1637, RIKEN Cell Bank, JPN), NIH3T3-3
15 (RCB0150, RIKEN Cell Bank, JPN), HeLa (RCB0007, RIKEN Cell
Bank, JPN), and HepG2 (RCB1648, RIKEN Cell Bank, JPN). In
the case of human MSCs, cells were maintained in
commercialized Human Mesenchymal Cell Basal Medium (MSCGM
BulletKit PT-3001, Cambrex BioScience Walkersville, Inc.,
20 MD). In case of HEK293, NIH3T3-3, HeLa and HepG2, cells were
maintained in Dulbecco's Modified Eagle's Medium (DMEM, high
glucose 4.5 g/L with L-Glutamine and sodium pyruvate;
14246-25, Nakalai Tesque, JPN) with 10% fetal bovine serum
(FBS, 29-167-54, Lot No. 2025F, Dainippon Pharmaceutical
25 CO., LTD., JPN). All cells were cultivated in a controlled
incubator at 37°C in 5% CO₂. In experiments involving hMSCs,
we used hMSCs of less than five passages, in order to avoid
phenotypic changes.

30 (Plasmids and Transfection reagents)

To evaluate the efficiency of transfection, the
pEGFP-N1 and pDsRed2-N1 vectors (cat. no. 6085-1, 6973-1,
BD Biosciences Clontech, CA) were used. Both genes'

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expressions were under the control of cytomegalovirus (CMV) promoter. Transfected cells continuously expressed EGFP or DsRed2, respectively. Plasmid DNAs were amplified using Escherichia coli, XL1-blue strain (200249, Stratagene, TX), and purified by EndoFree Plasmid Kit (EndoFree Plasmid Maxi Kit 12362, QIAGEN, CA). In all cases, plasmid DNA was dissolved in DNase and RNase free water. Transfection reagents were obtained as below: Effectene Transfection Reagent (cat. no. 301425, Qiagen, CA), TransFast™ Transfection Reagent (E2431, Promega, WI), Tfx™-20 Reagent (E2391, Promega, WI), SuperFect Transfection Reagent (301305, Qiagen, CA), PolyFect Transfection Reagent (301105, Qiagen, CA), LipofectAMINE 2000 Reagent (11668-019, Invitrogen corporation, CA), JetPEI (×4) conc. (101-30, Polyplus-transfection, France), and ExGen 500 (R0511, Fermentas Inc., MD).

(Solid-Phase Transfection Array (SPTA) production)

The detail of protocols for 'reverse transfection' was described in the web site, 'Reverse Transfection Homepage' (http://staffa.wi.mit.edu/sabatini_public/reverse_transfection.htm) or J. Ziauddin, D. M. Sabatini, Nature, 411, 2001, 107; and R.W. Zu, S.N. Bailey, D.M. Sabatini, Trends in Cell Biology, Vol. 12, No. 10, 485. In our solid phase transfection (SPTA method), three types of glass slides were studied (silanized glass slides; APS slides, and poly-L-lysine coated glass slides; PLL slides, and MAS coated slides; Matsunami Glass, JPN) with a 48 square pattern (3 mm × 3 mm) separated by a hydrophobic fluoride resin coating.

(Plasmid DNA printing solution preparation)

Two different ways to produce a SPTA were developed.

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The main differences reside in the preparation of the plasmid DNA printing solution.

(Method A)

5 In the case of using Effectene Transfection Reagent, the printing solution contained plasmid DNA and cell adhesion molecules (bovine plasma fibronectin (cat. no. 16042-41, Nakalai Tesque, JPN), dissolved in ultra-pure water at a concentration of 4 mg/mL). The above solution was applied
10 on the surface of the slide using an inkjet printer (synQUAD™, Cartesian Technologies, Inc., CA) or manually, using a 0.5 to 10 µL tip. This printed slide was dried up over 15 minutes at room temperature in a safety-cabinet. Before transfection, total Effectene reagent was gently poured on
15 the DNA-printed glass slide and incubated for 15 minutes at room temperature. The excess Effectene solution was removed from the glass slide using a vacuum aspirator and dried up at room temperature for 15 minutes in a safety-cabinet. The DNA-printed glass slide obtained was set in the bottom
20 of a 100-mm culture dish and approximately 25 mL of cell suspension (2 to 4×10^4 cells/mL) was gently poured into the dish. Then, the dish was transferred to the incubator at 37°C in 5% CO₂ and incubated for 2 or 3 days.

25 (Method B)

 In case of other transfection reagents (TransFast™, Tfx™-20, SuperFect, PolyFect, LipofectAMINE 2000, JetPEI (×4) conc., or ExGen), plasmid DNA, fibronectin, and the transfection reagent were mixed homogeneously in a 1.5-mL
30 micro-tube according to the ratios indicated in the manufacturer's instructions and incubated at room temperature for 15 minutes before printing on a chip. The printing solution was applied onto the surface of the

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glass-slide using an inkjet printer or a 0.5- to 10- μ L tip. The printed glass-slide was completely dried up at room temperature over 10 minutes in a safety-cabinet. The printed glass-slide was placed in the bottom of a 100-mm culture dish and approximately 3 mL of cell suspension (2 to 4 \times 10⁴ cells/mL) was added and incubated at room temperature over 15 minutes in a safety-cabinet. After incubation, fresh medium was poured gently into the dish. Then, the dish was transferred to an incubator at 37°C in 5% CO₂ and incubated for 2 to 3 days. After incubation, using fluorescence microscopy (IX-71, Olympus PROMARKETING, INC., JPN), we observed the transfectants, based on their expression of enhanced fluorescent proteins (EFP, EGFP and DsRed2). Phase contrast images were taken with the same microscope. In both protocols, cells were fixed by using a paraformaldehyde (PFA) fixation method (4% PFA in PBS, treatment time was 10 minutes at room temperature).

(Laser scanning and fluorescence intensity quantification)

In order to quantify the transfection efficiency, we used a DNA micro-array scanner (GeneTAC UC4 \times 4, Genomic Solutions Inc., MI). The total fluorescence intensity (arbitrary units) was measured, and thereafter, the fluorescence intensity per surface area was calculated.

(Results)

(Fibronectin-supported localized transfection)

A transfection array chip was constructed as shown in Figure 11. The transfection array chip was constructed by microprinting a cell cultivation medium solution containing fibronectin and DNA/transfection reagent onto a poly L lysine (PLL) coated glass slide.

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Various cells were used for this example. The cells were cultivated under typical cell cultivation conditions. As they adhered to the glass slide, the cells efficiently incorporated and expressed the genes corresponding to the DNA printed at a given position on the array. As compared to conventional transfection methods (e.g., cationic lipid or cationic polymer-mediated transfection), the efficiency of transfection using the method of the present invention was high in all the cells tested. Importantly, it was found that tissue stem cells, such as HepG2 and hMSC, which were conventionally believed to resist transfection, were efficiently transfected. hMSC was transfected at an efficiency 40 or more times higher than that of conventional techniques. In addition, high spatial localization, which is required for high-density arrays, was achieved (low cross contamination between adjacent spots on the array). This was confirmed by production of a checkered pattern array of EGFP and Ds-Red. hMSC cultivated on this array expressed the corresponding fluorescent proteins with virtually total space resolution. The result is shown in Figure 12. As can be seen from Figure 12, it was found that there was little cross contamination. Based on the study of the role of the individual components of the printed mixture, transfection efficiency can be optimized.

(Solid-phase transfection array of human mesenchymal stem cells)

The capacity of human Mesenchymal Stem Cells (hMSC) to differentiate into various kinds of cells is particularly intriguing in studies which target tissue regeneration and renewal. In particular, the genetic analysis of transformation of these cells has attracted attention with

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expectation of understanding of an agent that controls the pluripotency of hMSC. In conventional hMSC studies, it is not possible to perform transfection with desired genetic materials.

5

To achieve this, conventional methods include either a viral vector technique or electroporation. The present inventors developed a complex-salt system, which could be used to achieve solid phase transfection which makes it possible to obtain high transfection efficiency to various cell lines (including hMSC) and special localization in high-density arrays. An outline of solid phase transfection is shown in Figure 13A.

15

It was demonstrated that solid phase transfection can be used to achieve a "transfection patch" capable of being used for *in vivo* gene delivery and a solid phase transfection array (SPTA) for high-throughput genetic function research on hMSC.

20

Although a number of standard techniques are available for transfecting mammalian cells, it is known that it is inconvenient and difficult to introduce genetic material into hMSC as compared with cell lines, such as HEK293, HeLa, and the like. Conventional viral vector delivery and electroporation techniques are each important. However, these techniques have the following inconveniences: potential toxicity (for the virus technique); difficulty in high-throughput analysis at the genomic scale; and limited applications in *in vivo* studies (for electroporation).

30

The present inventors developed solid phase support fixed system which can be easily fixed to a solid phase support

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and has sustained-release capability and cell affinity, whereby most of the above-described drawbacks could be overcome.

5 An example of the results of the above-described experiment is shown in Figure 13B. The present inventors used our microprinting technique to fix a mixture of a selected genetic material, a transfection reagent, an appropriate cell adhesion molecule, and a salt onto a solid support.
10 By culturing cells on a support having such a mixture fixed thereonto, the gene contained in the mixture was allowed to be taken in by the cultured cells. As a result, it became possible to allow support-adherent cells to take in DNA spatially separated therefrom (Figure 13B).

15 As a result of this example, several important effects were achieved: high transfection efficiency (thereby making it possible to study a group of cells having a statistically significant scale); low cross contamination between regions
20 having different DNA molecules (thereby making it possible to study the effects of different genes separately); the extended survival of transfected cells; high-throughput, compatible and simple detecting procedure. SPTA having these features serves as an appropriate basis for further
25 studies.

 To achieve the above-described objects, the present inventors studied five different cell lines (HEK293, HeLa, NIH3T3, HepG2 and hMSC) as described above with both our
30 methodology (transfection in a solid phase system) (see Figures 13A and 13C) and conventional liquid-phase transfection under a series of transfection conditions. Cross contamination was evaluated for both systems as follows.

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In the case of SPTA, we printed DNA's encoding a red fluorescent protein (RFP) and a green fluorescent protein (GFP) on glass supports in a checked pattern. In the case of experiments including conventional liquid phase transfection (where
5 cells to be transfected cannot be spatially separated from one another spontaneously), a DNA encoding GFP was used. Several transfection reagents were evaluated: four liquid transfection reagents (Effectene, TransFast™, Tfx™-20, LopofectAMINE 2000), two polyamine (SuperFect, PolyFect),
10 and two polyimine (JetPEI (x4) and ExGen 500).

Transfection efficiency: transfection efficiency was determined as total fluorescence intensity per unit area (Figure 14A and Figure 14B (images)). The results of liquid
15 phase optimal to cell lines used were obtained using different transfection reagents (see Figures 14C to 14D). Next, these efficient transfection reagents were used to optimize a solid phase protocol. Several tendencies were observed. For cell lines which are readily transfectable (e.g., HEK293, HeLa,
20 NIH3T3, etc.), the transfection efficiency observed in the solid phase protocol was slightly superior to, but essentially similar to, that of the standard liquid phase protocol (Figure 14).

25 However, for cells which are difficult to transfect (e.g., hMSC, HepG2, etc.), we observed that transfection efficiency was increased up to 40 fold while the features of the cells were retained under conditions optimized to the SPTA methodology (see the above-described protocol and
30 Figures 14C and 14D). In the case of hMSC (Figure 15), the best conditions included use of a polyethylene imine (PEI) transfection reagent. As expected, important factors for achieving high transfection efficiency are the charge balance

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(N/P ratio) between the number of nitrogen atoms (N) in the polymer and the number of phosphate residues (P) in plasmid DNA and DNA concentration. Generally, increases in the N/P ratio and the concentration lead to an increase in transfection efficiency. We also observed a significant reduction in the survival rate of hMSC cells in liquid phase transfection experiments where the DNA concentration was high and the N/P ratio was high. Because of these two opposing factors, the liquid phase transfection of hMSC had a relatively low cell survival rate (N/P ratio >10). In the case of the SPTA protocol, however, a considerably high N/P ratio (fixed to the solid support) and DNA concentration were tolerable (probably attributed to the effect of the solid support stabilizing cell membrane) while the cell survival rate and the cellular state were not significantly affected. Therefore, this is probably responsible for the dramatic improvement in transfection efficiency. It was found that the N/P ratio of 10 was optimal for SPTA, and a sufficient transfection level was provided while minimizing cytotoxicity. Another reason for the increase in transfection efficiency observed in the case of the SPTA protocol is that a high local ratio of the DNA concentration to the transfection reagent concentration was achieved (this leads to cell death in liquid phase transfection experiments).

A coating agent used is crucial for the achievement of high transfection efficiency on chips. It was found that when a glass chip is used, PLL provided best results both for transfection efficiency and cross contamination (described below). When fibronectin coating was not used, few transfectants were observed (all the other experimental conditions were retained unchanged). Although not

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completely established, fibronectin probably plays a role in accelerating cell adhesion process (data not shown), and thus, limiting the time which permits the diffusion of DNA released from the surface.

5

Low cross contamination: apart from the higher transfection efficiency observed in the SPTA protocol, an important advantage of the technique of the present invention is to achieve an array of separated cells, in which selected genes are expressed in the separate positions. The present inventors printed JetPEI (see the "Experimental protocols" section) and two different reporter genes (RFP and GFP) mixed with fibronectin on glass surface coated with fibronectin. The resultant transfection chip was subjected to appropriate cell culture. Expressed GFP and RFP were localized in regions, in which corresponding cDNA had been spotted, under experimental conditions which had been found to be best. Substantially no cross contamination was observed (Figure 16). In the absence of fibronectin or PLL, however, cross contamination which hinders solid phase transfection was observed, and the transfection efficiency was significantly lower (see Figure 6). This result demonstrated the hypothesis that the relative proportion of plasmid DNA, which was released from the cell adhesion and the support surface, is a factor important for high transfection efficiency and high cross contamination.

Another cause of cross contamination may be the mobility of transfected cells on a solid support. The present inventors measured both the rate of cell adhesion (Figure 16C) and the diffusion rate of plasmid DNA on several supports. As a result, substantially no DNA diffusion occurred under optimum conditions. However, a considerably

30

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amount of plasmid DNA were diffused under high cross contamination conditions until cell adhesion was completed, so that plasmid DNA was depleted from the solid phase surface.

5 This established technique is of particular importance in the context of cost-effective high-throughput gene function screening. Indeed, the small amounts of transfection reagent and DNA required, as well as the possible automatization of the entire process (from plasmid isolation
10 to detection) increase the utility of the above presented method.

 In conclusion, the present invention successfully realized a hMSC transfection array in a system using
15 complex-salt. With this technique, it will be possible to achieve high-throughput studies using the solid phase transfection, such as the elucidation of the genetic mechanism for differentiation of pluripotent stem cells. The detailed mechanism of the solid phase transfection as
20 well as methodologies for the use of this technology for high throughput, real time gene expression monitoring can be applied for various purposes.

(Example 5: RNAi transfection microarray)

25 Arrays were produced as described in the above-described example. As genetic material, mixtures of plasmid DNA (pDNA) and shRNA were used. The compositions of the mixtures are shown in Table 2.

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Table 2

	pDNA vs. shRNA ratio [μ L/ μ L]				
	9:1	7:3	1:1	3:7	1:9
pEGFP-N1 (1 mg/mL)	1.8	1.4	1.0	0.6	0.2
pPUR61GFP272 (1 mg/mL)	0.2	0.6	1.0	1.4	1.8
pDsRed2-1 (1 mg/mL)	0.2	0.6	1.0	1.4	1.8
Lipofectamine2000	4.0	4.0	4.0	4.0	4.0
Fibronectin (4 mg/mL)	5.0	5.0	5.0	5.0	5.0

The results are shown in Figure 17. For each of the 5 cells, the results of Figure 17 are converted into numerical data in Figures 18A to 18E.

Thus, it was revealed that the method of the present invention is applicable to any cells.

10 (Example 6: Use of RNAi microarray=siRNA)

Next, siRNA was used instead of shRNA to construct RNAi transfection microarrays in accordance with a protocol as described in the above-described example.

15 18 transcription factor reporters and actin promoter vectors described in Table 3 were used to synthesize 28 siRNAs for the transcription factors. siRNA for EGFP was used as a control. Each siRNA was evaluated as to whether or not it knocks out a target transcription factor. Scramble RNAs
20 were used as negative controls, and their ratios were evaluated.

Table 3

Mercury signaling pathway
pAP1(PMA)-EGFP
pAP1-EGFP
pCRE-EGFP
pERE-EGFP
pE2F-EGFP
pGAS-EGFP
pGRE-EGFP
pHSE-EGFP
pISRE-EGFP
pMyc-EGFP
pNFAT-EGFP
pNFkB-EGFP
p53-EGFP
pRARE-EGFP
pRb-EGFP
pSRE-EGFP
pSTAT3-EGFP
pTRE-EGFP

Each cell was subjected to solid phase transfection, followed by culture for two days. Images were taken using a fluorescence image scanner, and the fluorescent level was quantified.

The results are shown in Figure 19. The results were summarized for each gene in Figures 20A to 20D.

As shown in Figures 19 and 20A to 20D, when RNAi was used, the expression of each gene was specifically suppressed. Thus, it was demonstrated that an array having a plurality of genetic materials, which is applicable to RNAi, can be realized and time-lapse analysis can be performed for the effect of RNAi on cells.

(Example 7: Transfection array using PCR fragments)

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Next, it was demonstrated that the present invention could be implemented when PCR fragments were used as genetic materials. The procedure will be described below.

5 PCR was performed to obtain nucleic acid fragments as shown in Figure 21. These fragments were used as genetic materials which were applied to transfection microarrays. The procedure will be described below.

10 PCR primers were:
GG ATAACCGTAT TACCGCCATG CAT (SEQ ID NO.: 12); and
ccctatctcgggtctattcttttg CAAAAGAATA GACCGAGATA GGG
(SEQ ID NO.: 13).

15 pEGFP-N1 (see Figure 22) was used as a template.

PCR conditions were described in Table 4 below.

Table 4

Distilled water	33.5	μL
10×KOD-Plus-buffer	5	μL
2 mM dNTPs	5	μL
25 mM MgSO ₄	2	μL
Primer (10 μM each)	1.5	μL
Template DNA (1 ng)	2	μL
KOD-Plus-(1unit/μL)	1	μL
Total	50	μL

20

Cycle conditions: 94°C, 2 min → (94°C, 15 sec → 60°C, 30 sec → 68°C, 3 min) → 4°C (the process in parenthesis was performed 30 times)

25

The resultant PCR fragment was purified with

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phenol/chloroform extraction and ethanol precipitation.
The PCR fragment has the following sequence:

GG ATAACCGTAT TACCGCCATG CAT TAGTTATTAA TAGTAATCAA TTACGGGGTC
ATTAGTTCAT AGCCCATATA TGGAGTTCCG
5 CGTTACATAA CTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCATT
GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA
ATGGGTGGAG TATTTACGGT AAAGTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCAGTA
CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC
10 CATGGTGATG CGGTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG
ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC AAAATCAACG
GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT
ACGGTGGGAG GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC GCTAGCGCTA
CCGGACTCAG ATCTCGAGCT CAAGCTTCGA ATTCTGCAGT CGACGGTACC GCGGGCCCCG
15 GATCCACCGG TCGCCACCAT GGTGAGCAAG GGCGAGGAGC TGTTACCAGG GGTGGTGCCC
ATCCTGGTCG AGCTGGACGG CGACGTAAAC GGCCACAAGT TCAGCGTGTC CGGCGAGGGC
GAGGGCGATG CCACCTACGG CAAGCTGACC CTGAAGTTCA TCTGCACCAC CGGCAAGCTG
CCCGTGCCCT GGCCACCCCT CGTGACCACC CTGACCTACG GCGTGCAGTG CTTAGCCGC
TACCCCGACC ACATGAAGCA GCAGGACTTC TTCAAGTCCG CCATGCCCGA AGGCTACGTC
20 CAGGAGCGCA CCATCTTCTT CAAGGACGAC GGCAACTACA AGACCCGCGC CGAGGTGAAG
TTGAGGGCG ACACCCTGGT GAACCGCATC GAGCTGAAGG GCATCGACTT CAAGGAGGAC
GGCAACATCC TGGGGCACA GCTGGAGTAC AACTACAACA GCCACAACGT CTATATCATG
GCCGACAAGC AGAAGAACGG CATCAAGGTG AACTTCAAGA TCCGCCACAA CATCGAGGAC
GGCAGCGTGC AGCTCGCCGA CCACTACCAG CAGAACACCC CCATCGGCGA CGGCCCCGTG
25 CTGCTGCCCC ACAACCACTA CCTGAGCACC CAGTCCGCCC TGAGCAAAGA CCCCACGAG
AAGCGCGATC ACATGGTCCT GCTGGAGTTC GTGACCGCCG CCGGGATCAC TCTCGGCATG
GACGAGCTGT ACAAGTAAAG CGGCCGCGAC TCTAGATCAT AATCAGCCAT ACCACATTTG
TAGAGGTTTT ACTTGCTTTA AAAAACCTCC CACACCTCCC CCTGAACCTG AAACATAAAA
TGAATGCAAT TGTTGTTGTT AACTTGTTTA TTGCAGCTTA TAATGGTTAC AAATAAAGCA
30 ATAGCATCAC AAATTTTACA AATAAAGCAT TTTTTTCACT GCATTCTAGT TGTGGTTTGT
CCAACTCAT CAATGTATCT TAAGGCGTAA ATTGTAAGCG TTAATATTTT GTTAAAATTC
GCGTTAAATT TTTGTAAAT CAGCTCATTT TTTAACCAAT AGGCCGAAAT CGGCAAAATC
CCTTATAAAT CAAAAGAATA GACCGAGATA GGG (SEQ ID NO.: 14).

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Chips were produced using the PCR fragment. MCF7 was disseminated on the chips. After two days, images were obtained using a fluorescence image scanner. The results are shown in Figure 23. In Figure 23, the PCR fragment is compared with circular DNA. In either case, transfection was successful. It was revealed that the PCR fragment, which was used as a genetic material, could be transfected into cells, as with full-length plasmids, so that time-lapse analysis could be performed for the cells. Thus, the fixing effect of the salt and the enhancement of gene introduction by such an effect were confirmed.

(Example 8: Type of support)

Next, when a solid phase support is made of silica, silicon, a ceramic, silicon dioxide, or a plastic instead of glass, it is determined whether or not a similar effect of actin acting substances is observed.

These materials are available from Matsunami Glass. Arrays are produced as described above.

As a result, it is revealed that a similar effect of actin can be observed for the material used.

(Example 9: Regulation of gene expression using tetracycline-dependent promoter)

As described in the above-described examples, it was demonstrated that a tetracycline-dependent promoter could be used to produce a profile showing how gene expression is regulated. The sequences described below were used.

As the tetracycline-dependent promoter (and its gene

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vector construct), pTet-Off and pTet-On vectors (BD Biosciences) were used (see <http://www.clontech.com/techinfo/vectors/cattet.shtml>). As a vector, pTRE-d2EGFP (SEQ ID NO.: 18) was used (see <http://www.clontech.com/techinfo/vectors/vectorsT-Z/pTRE-d2EGFP.shtml>).

- 5 pTet-Off (BD Clontech K1620-A)
- Fragment containing P_{CMV} : 86-673
 - 10 · Tetracycline-responsive transcriptional activator (tTA): 774-1781
 - Col E1 origin of replication: 2604-3247
 - ? Ampicillin resistance gene:
 - β-lactamase coding sequences: 4255-3395
 - 15 · Fragment containing the SV40 poly A signal: 1797-2254
 - Neomycin/kanamycin resistance gene: 6462-5668
 - SV40 promoter (P_{SV40}) controlling expression of neomycin/kanamycin resistance gene: 7125-6782.
- 20 pTet-ON(BD Clontech K1621-A)
- Fragment containing P_{CMV}: 86-673
 - Reverse tetracycline-responsive transcriptional activator (rtTA): 774-1781
 - pUC origin of replication: 2604-3247
 - 25 · Ampicillin resistance gene:
 - β-lactamase coding sequences: 4255-3395
 - Fragment containing the SV40 poly A signal: 1797-2254
 - Neomycin/kanamycin resistance gene: 6462-5668
 - SV40 promoter (P_{SV40}) controlling expression of
 - 30 neomycin/kanamycin resistance gene: 7125-6782.

pTRE-d2EGFP(BD Clontech 6242-1)

- P_{hCMV*-1} Tet-responsive promoter: 1-438

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- Tet-responsive element (TRE): 1-318
 - Location of seven *tetO*18-mers: 15-33; 57-75; 99-117; 141-159; 183-201; 225-243; & 257-275
 - Fragment containing P_{minCMV} : 319-438
- 5 TATA box 341-348
- Destabilized enhanced green fluorescent protein (d2EGFP) gene
 - Start codon: 445-447; stop codon: 1288-1290
 - Insertion of Val at position #2: 448-450
- 10 GFPmut1 mutations (Phe-64-Leu, Ser-65-Thr): 634-639
- His-231-Leu: 1137
 - Mouse ornithine decarboxylase (MODC) PEST sequence: 1167-1290
- 15 Fragment containing SV40 poly A signal: 1330-1787
- (approximate coordinates of poly A signal: 1448-1453)
- Fragment containing Col E1 origin of replication: 2137-2780
- 20 Ampicillin resistance gene
- β -lactamase coding sequences: 2928-3788
 - start codon: 3788-3786
 - stop codon: 2928-2930
- 25 (Protocol)
- pTet-Off and pTet-On (SEQ ID NOS.: 15 and 16, respectively) were printed onto array substrates. Realtime measurement was performed on the array substrates to determine whether or not tetracycline regulates gene
- 30 expression. The results are shown in Figure 24. As shown in Figure 24, a change in gene expression was detected only for the tetracycline-dependent promoter. Figure 25 is a photograph showing the actual states of expression for the

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tetracycline-dependent promoter and the tetracycline-independent promoter. As can be seen, the difference between them is measurable by the naked eye.

5 Although certain preferred embodiments have been described herein, it is not intended that such embodiments be construed as limitations on the scope of the invention except as set forth in the appended claims. Various other
10 modifications and equivalents will be apparent to and can be readily made by those skilled in the art, after reading the description herein, without departing from the scope and spirit of this invention. All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein.

15

INDUSTRIAL APPLICABILITY

 According to the present invention, transfection efficiency could be increased either in a solid phase and
20 in a liquid phase. The reagent for increasing transfection efficiency is useful for transfection in, particularly, solid phases.

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CLAIMS

1. A composition for increasing the efficiency of introducing
a target substance into a cell, comprising:
 - 5 (a) an actin acting substance.
2. A composition according to claim 1, wherein the actin
acting substance may be an extracellular matrix protein or
a variant or fragment thereof.
- 10 3. A composition according to claim 2, wherein the actin
acting substance comprises at least one protein selected
from the group consisting of fibronectin, laminin, and
vitronectin, or a variant or fragment thereof.
- 15 4. A composition according to claim 1, wherein the actin
acting substance comprises:
 - (a-1) a protein molecule comprising at least amino
acids 21 to 241 of SEQ ID NO.: 11 constituting an Fn1 domain,
20 or a variant thereof;
 - (a-2) a protein molecule having an amino acid
sequence set forth in SEQ ID NO.: 2 or 11, or a variant or
fragment thereof;
 - (b) a polypeptide having an amino acid sequence set
25 forth in SEQ ID NO.: 2 or 11 having at least one mutation
selected from the group consisting of at least one amino
acid substitution, addition, and deletion, and having a
biological activity;
 - (c) a polypeptide encoded by a splice or allelic mutant
30 of a base sequence set forth in SEQ ID NO.: 1;
 - (d) a polypeptide being a species homolog of the amino
acid sequence set forth in SEQ ID NO.: 2 or 11; or
 - (e) a polypeptide having an amino acid sequence

having at least 70% identity to any one of the polypeptides (a-1) to (d), and having a biological activity.

5 5. A composition according to claim 1, wherein the Fnl domain comprises amino acids 21 to 577 of SEQ ID NO.: 11.

6. A composition according to claim 1, wherein the protein molecule having the Fnl domain is fibronectin or a variant or fragment thereof.

10 7. A composition according to claim 1, further comprising a gene introduction reagent.

15 8. A composition according to claim 1, wherein the gene introduction reagent is selected from the group consisting of cationic polymers, cationic lipids, and calcium phosphate.

9. A composition according to claim 1, further comprising a particle.

20 10. A composition according to claim 9, wherein the particle comprises gold colloid.

25 11. A composition according to claim 1, further comprising a salt.

12. A composition according to claim 11, wherein the salt is selected from the group consisting of salts contained in buffers and salts contained in media.

30 13. A kit for increasing the efficiency of introducing a target substance into a cell, comprising:

(a) a composition comprising an actin acting

- 129 -

substance; and

(b) a gene introduction reagent.

14. A composition for increasing the efficiency of
5 introducing a target substance into a cell, comprising:

A) a target substance; and

B) an actin acting substance.

15 15. A composition according to claim 14, wherein the target
substance comprises a substance selected from the group
consisting of DNA, RNA, polypeptides, sugars, and complexes
thereof.

16 16. A composition according to claim 14, wherein the target
substance comprises DNA encoding a gene sequence to be
transfected.

17 17. A composition according to claim 16, further comprising
a gene introduction reagent.

20 18. A composition according to claim 14, wherein the actin
acting substance is an extracellular matrix protein or a
variant or fragment thereof.

25 19. A composition according to claim 14, wherein the
composition is provided in liquid phase.

20 20. A composition according to claim 14, wherein the
composition is provided in solid phase.

30 21. A device for introducing a target substance into a cell,
comprising:

A) a target substance; and

- 130 -

B) an actin acting substance,

wherein the composition is fixed to a solid phase support.

- 5 22. A device according to claim 21, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes thereof.
- 10 23. A device according to claim 21, wherein the target substance comprises DNA encoding a gene sequence to be transfected.
- 15 24. A device according to claim 23, further comprising a gene introduction reagent.
- 20 25. A device according to claim 21, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.
- 25 26. A device according to claim 21, wherein the solid phase support is selected from the group consisting of plates, microwell plates, chips, glass slides, films, beads, and metals.
- 30 27. A device according to claim 21, wherein the solid phase support is coated with a coating agent.
28. A device according to claim 27, wherein the coating agent comprises a substance selected from the group consisting of poly-L-lysine, silane, MAS, hydrophobic fluorine resins, and metals.

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29. A method for increasing the efficiency of introducing a target substance into a cell, comprising the steps of:

A) providing the target substance;

B) providing an actin acting substance; and

5 C) contacting the target substance and the actin acting substance with the cell.

30. A method according to claim 29, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes thereof.

10

31. A method according to claim 29, wherein the target substance comprises DNA encoding a gene sequence to be transfected.

15

32. A method according to claim 31, further comprising providing a gene introduction reagent, wherein the gene introduction reagent is contacted with the cell.

20

33. A method according to claim 29, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.

25 34. A method according to claim 29, wherein the steps are conducted in liquid phase.

35. A method according to claim 29, wherein the steps are conducted in solid phase.

30

36. A method for increasing the efficiency of introducing a target substance into a cell, comprising the steps of:

I) fixing a composition to a solid support, wherein

- 132 -

the composition comprising:

A) a target substance; and

B) an actin acting substance; and

5 II) contacting the cell with the composition on the solid support.

37. A method according to claim 36, wherein the target substance comprises a substance selected from the group consisting of DNA, RNA, polypeptides, sugars, and complexes
10 thereof.

38. A method according to claim 36, wherein the target substance comprises DNA encoding a gene sequence to be transfected.
15

39. A method according to claim 38, further comprising providing a gene introduction reagent, wherein the gene introduction reagent is contacted with the cell.

20 40. A method according to claim 39, further comprising forming a complex of the DNA and the gene introduction reagent after providing the gene introduction reagent, wherein after the forming step, the composition is provided by providing the actin acting substance.

25 41. A method according to claim 36, wherein the actin acting substance is an extracellular matrix protein or a variant or fragment thereof.

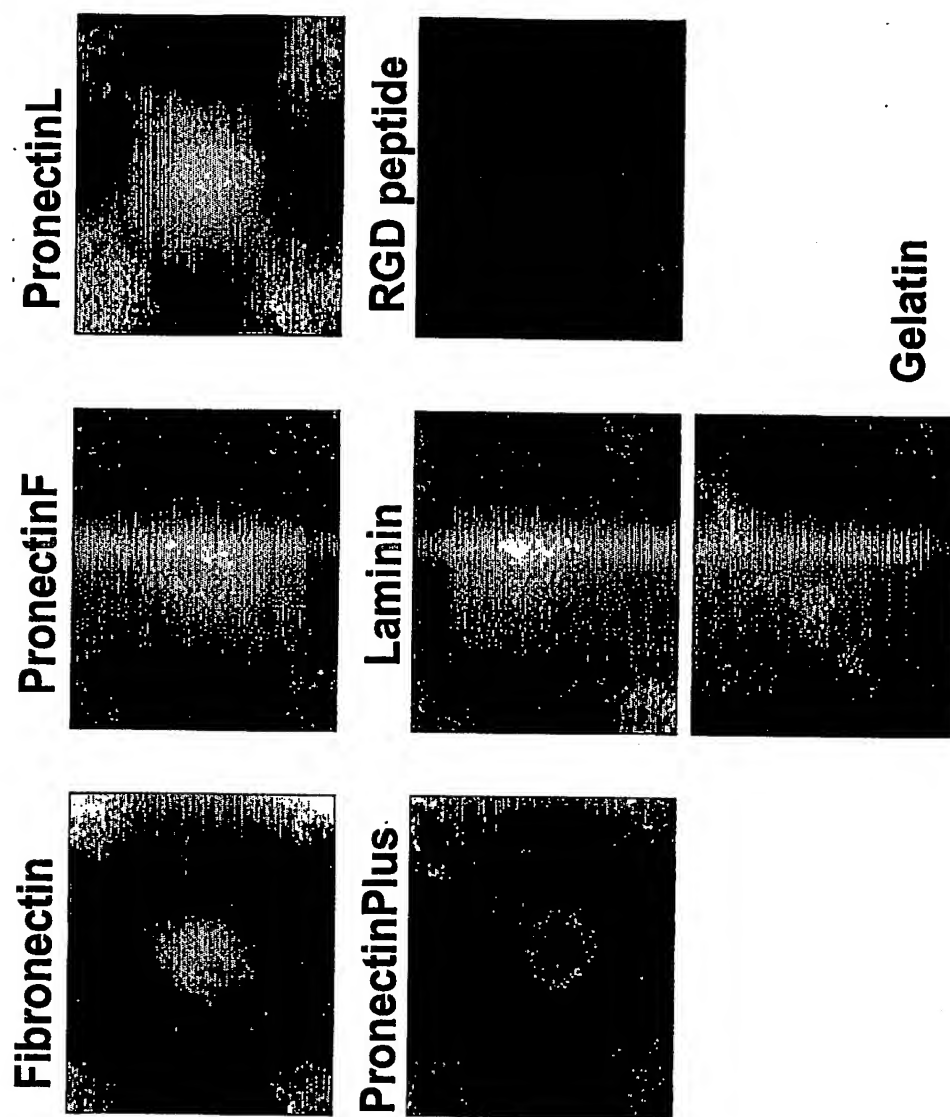
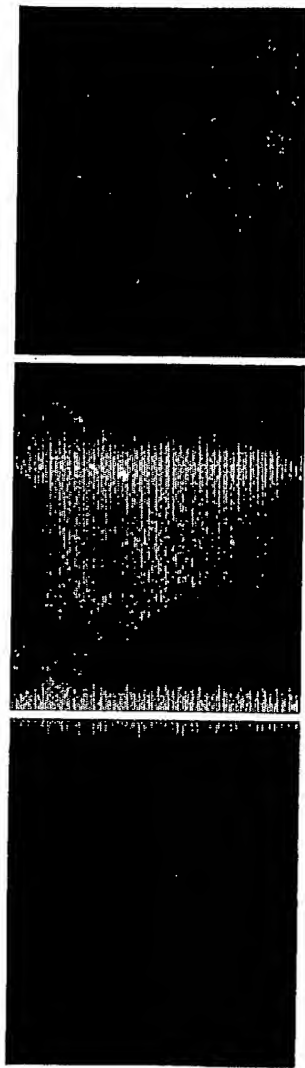


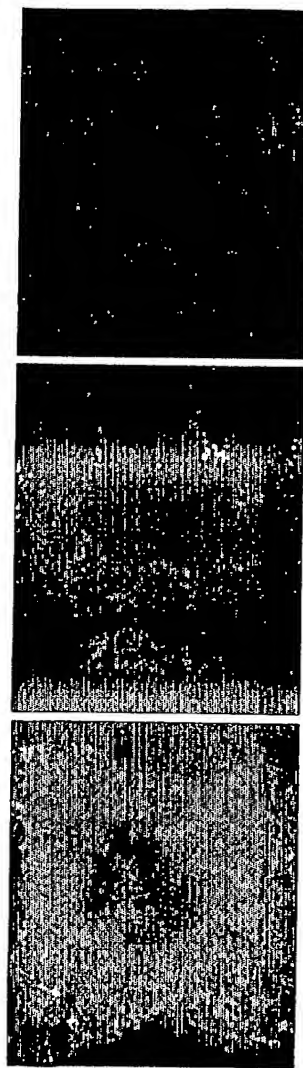
FIG.1

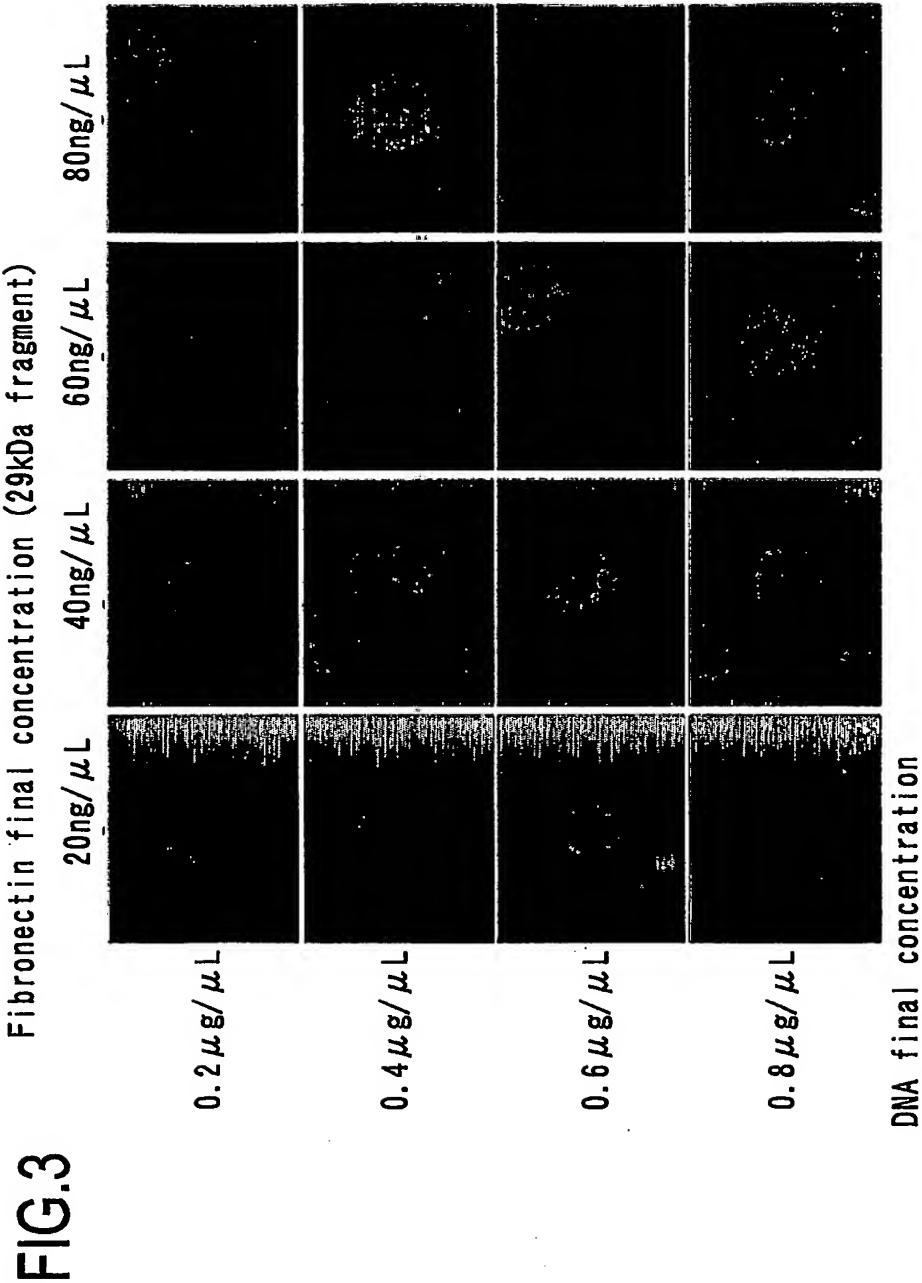
FIG.2

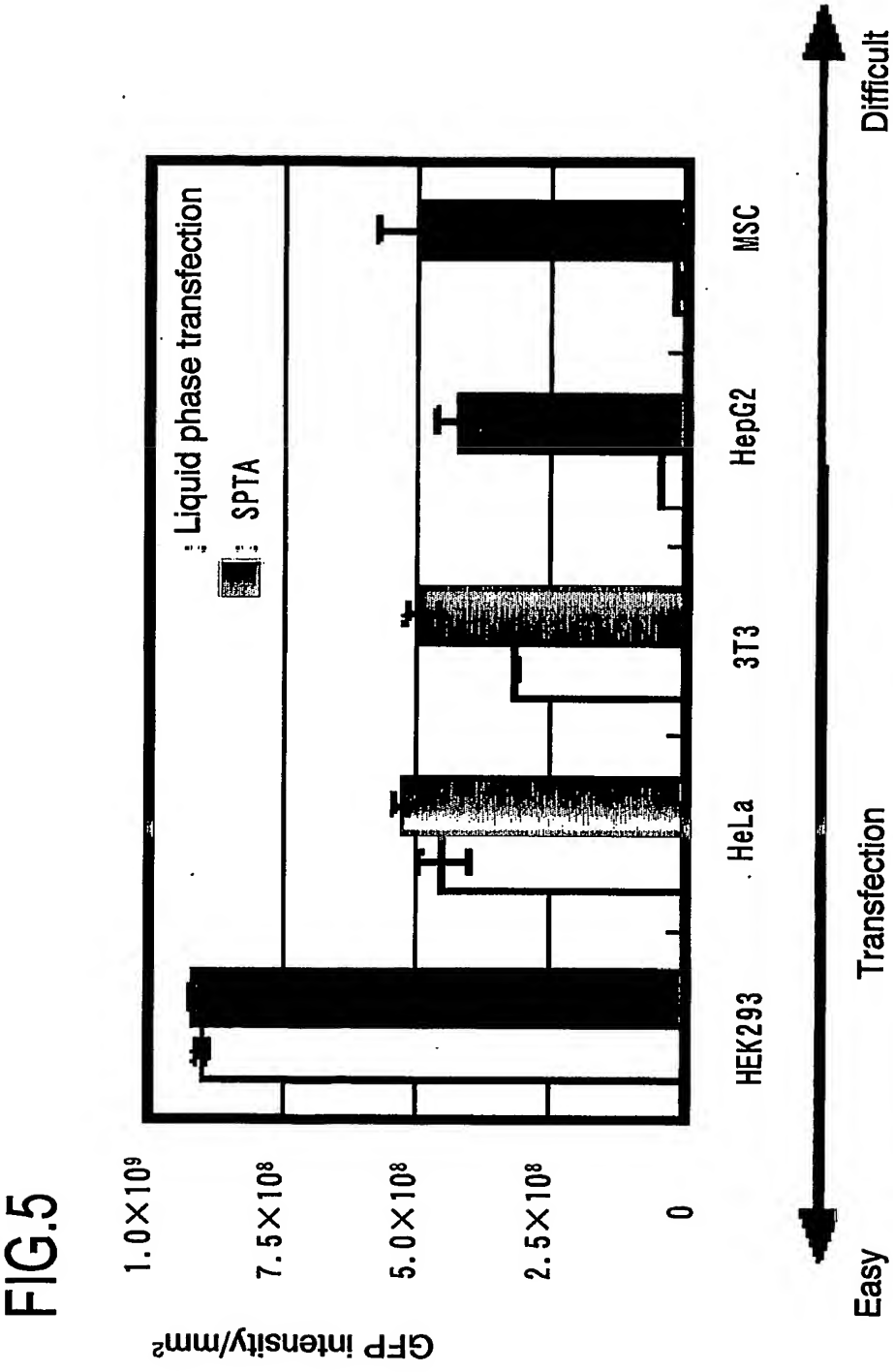
Fibronectin (43kDa fragment)

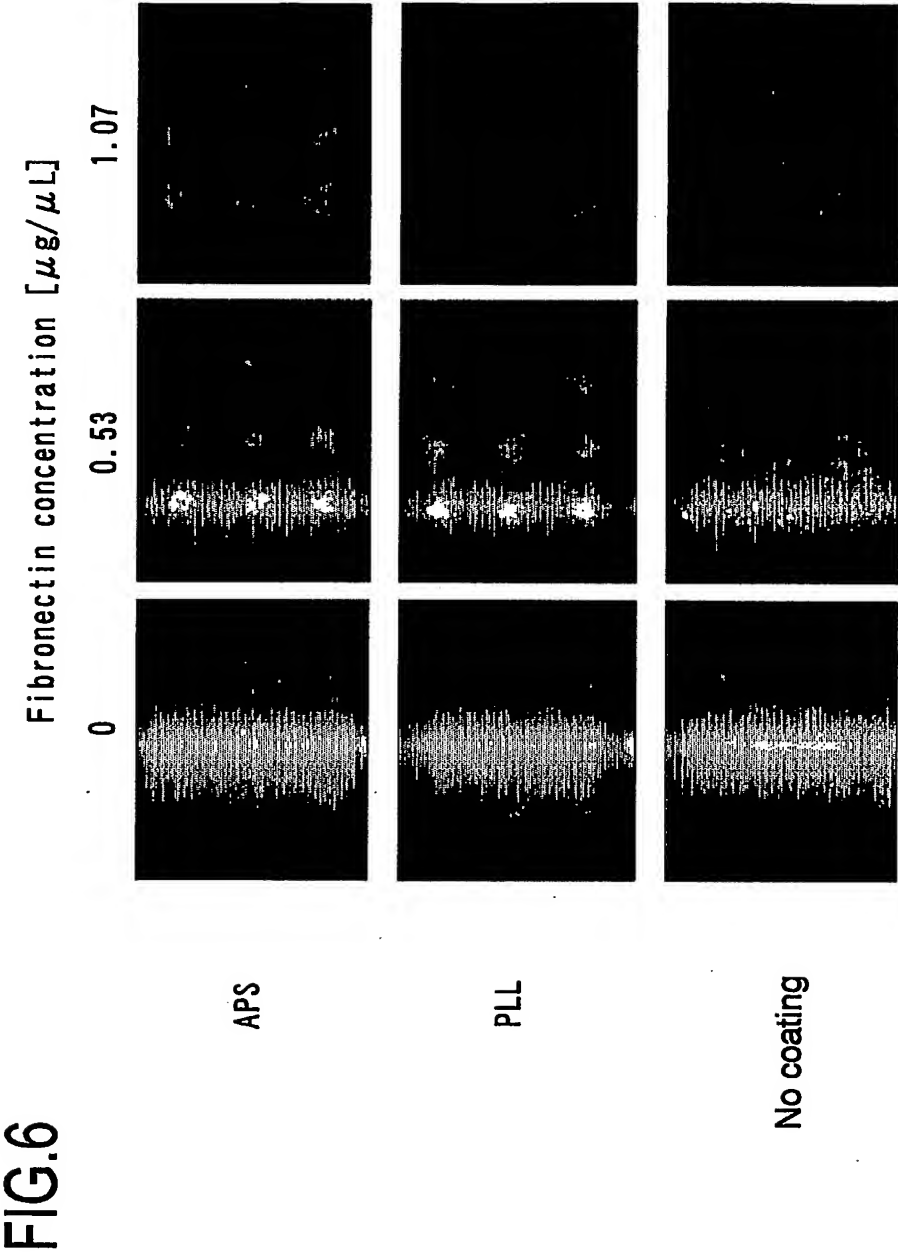


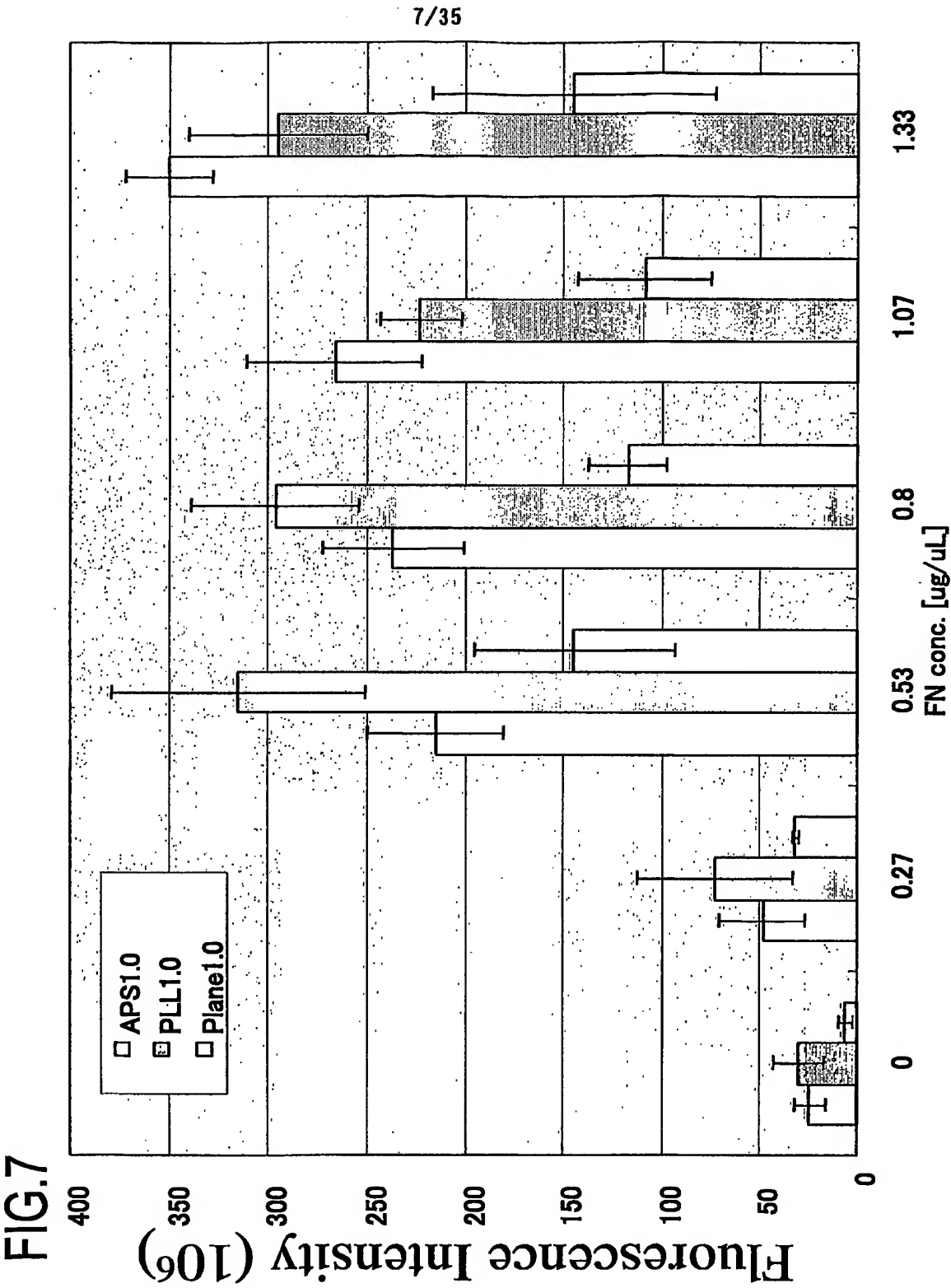
Fibronectin (72kDa fragment)





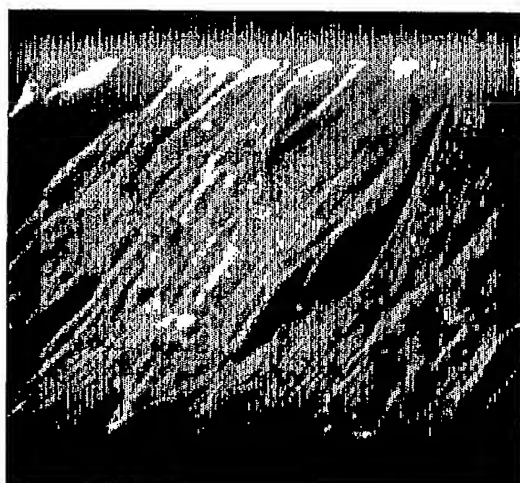




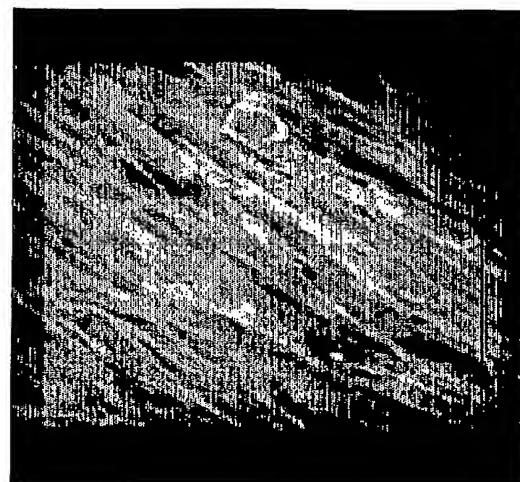


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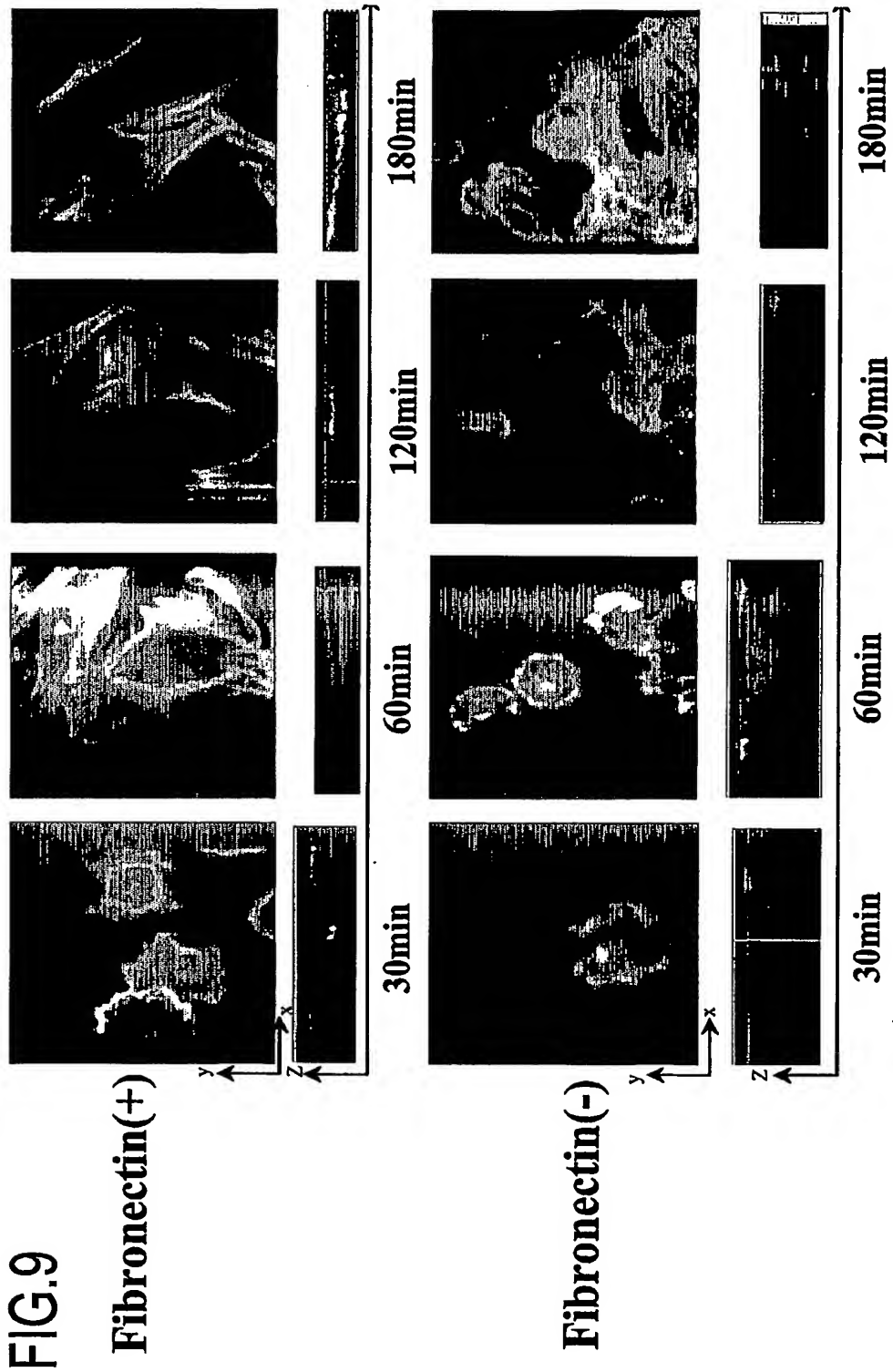
FIG.8



Fibronectin(+)



Fibronectin(-)



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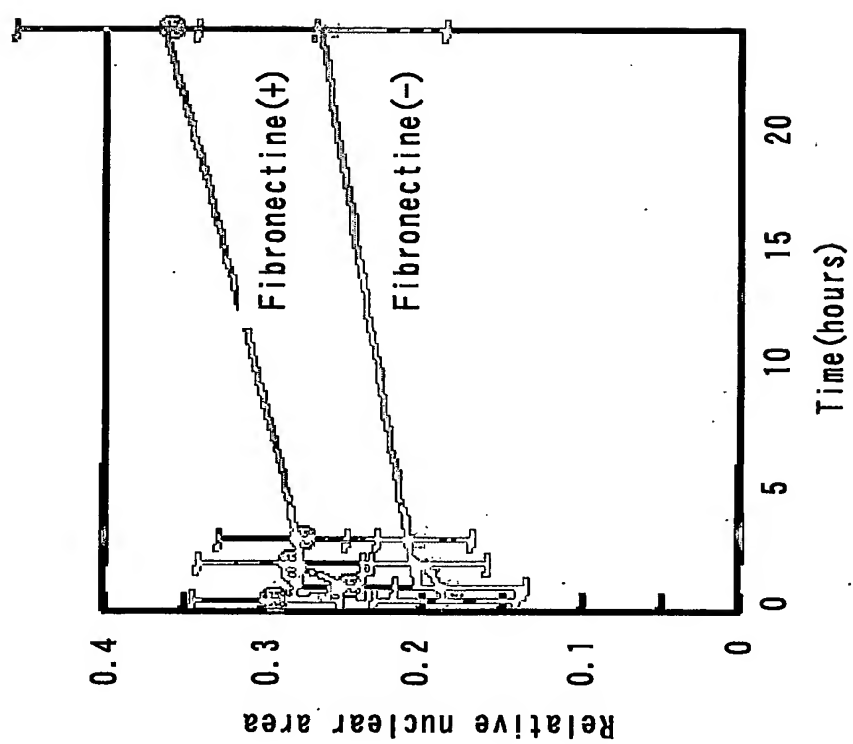
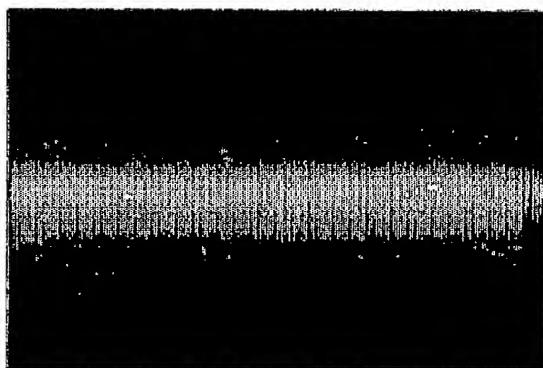


FIG.10

FIG.11



FIG.12



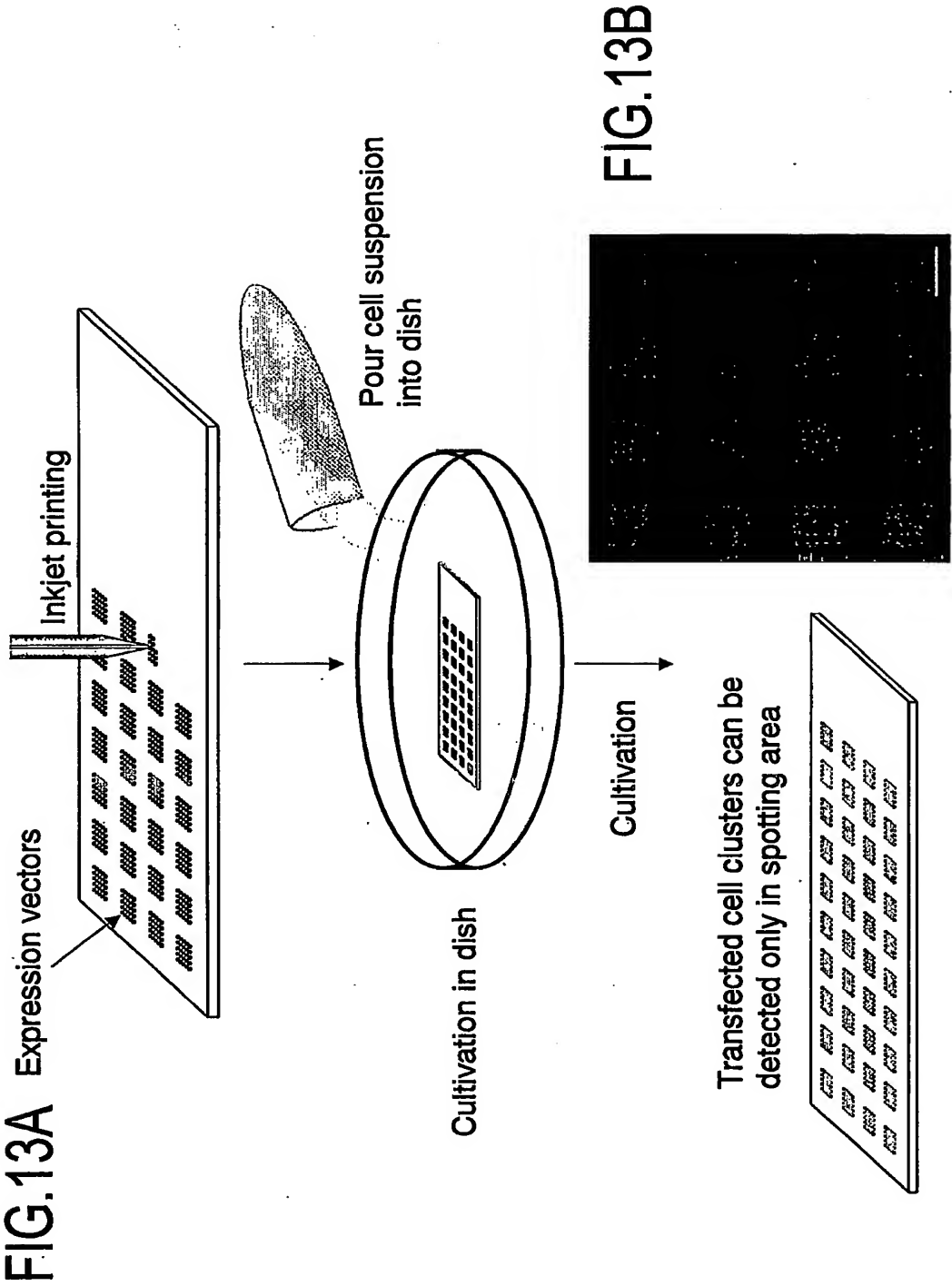
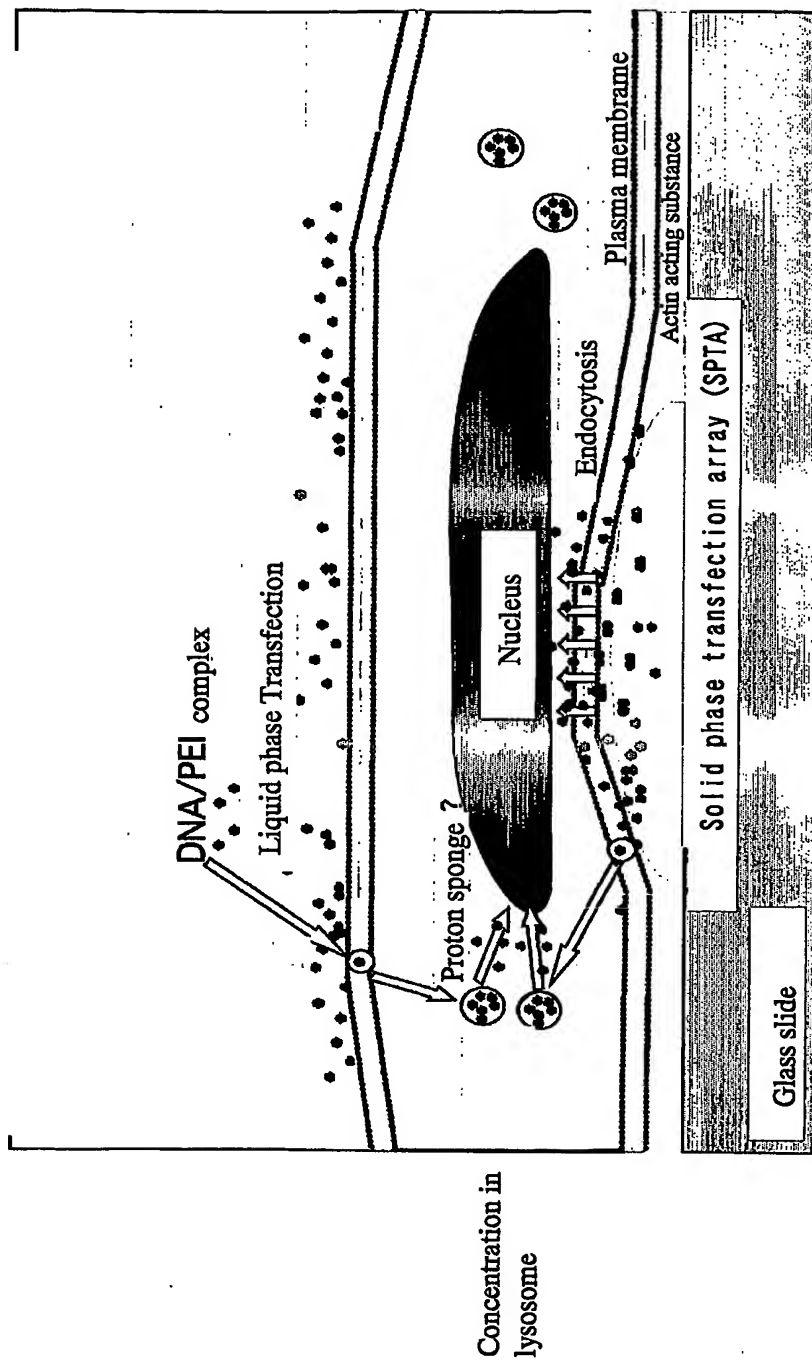
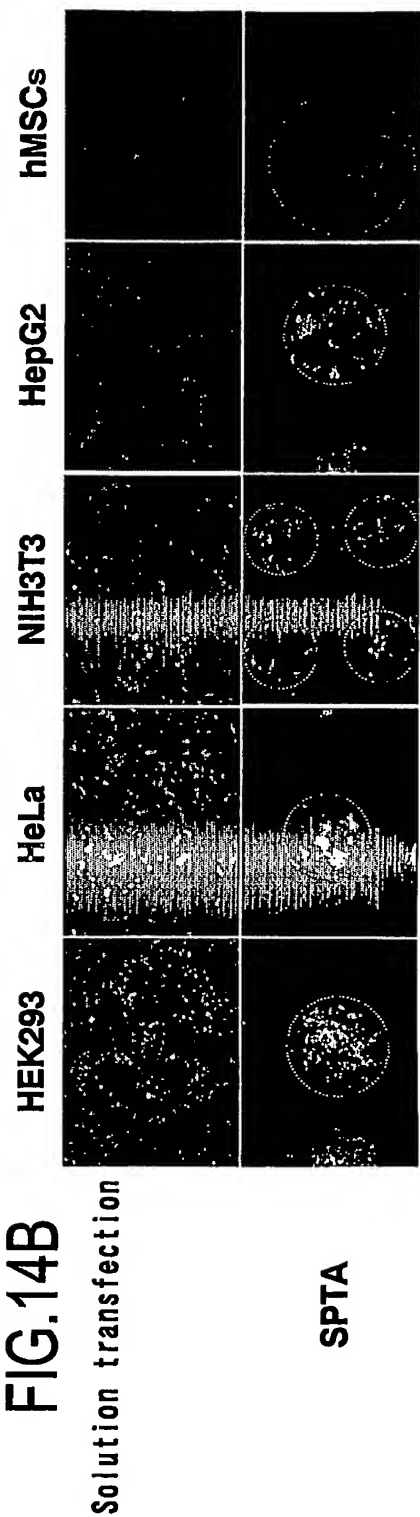
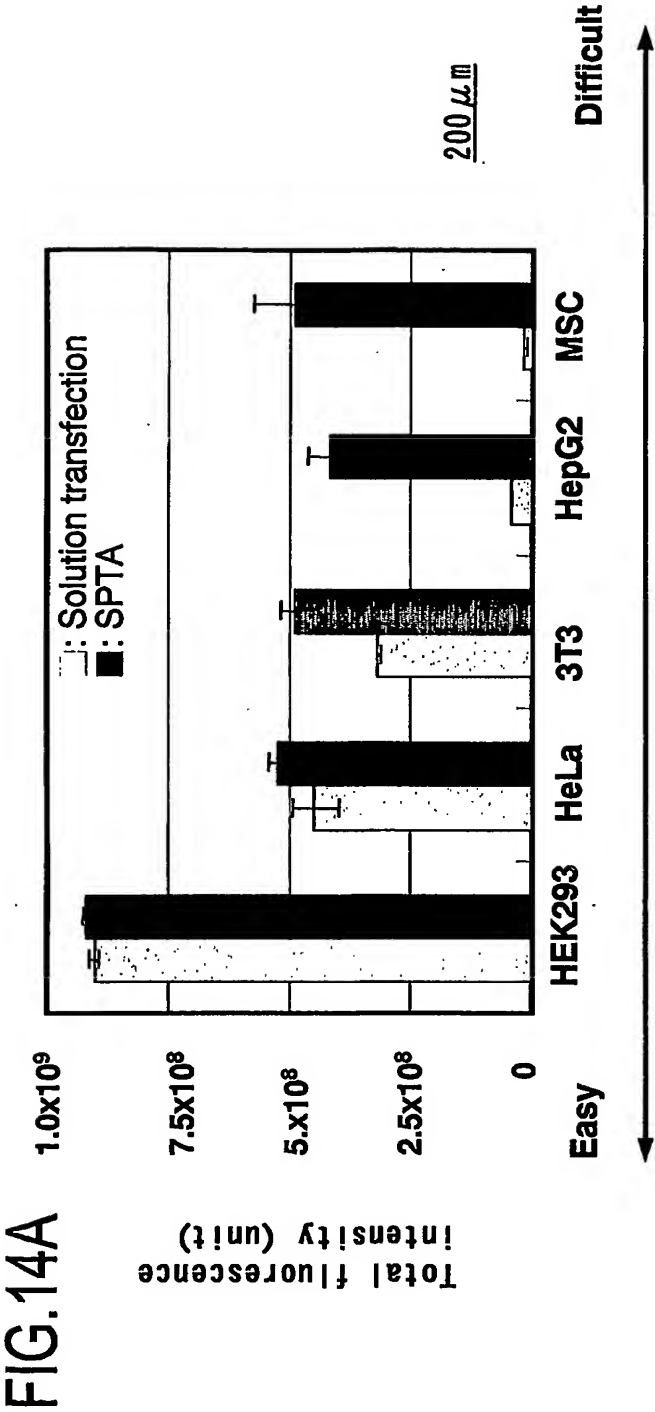


FIG. 13C





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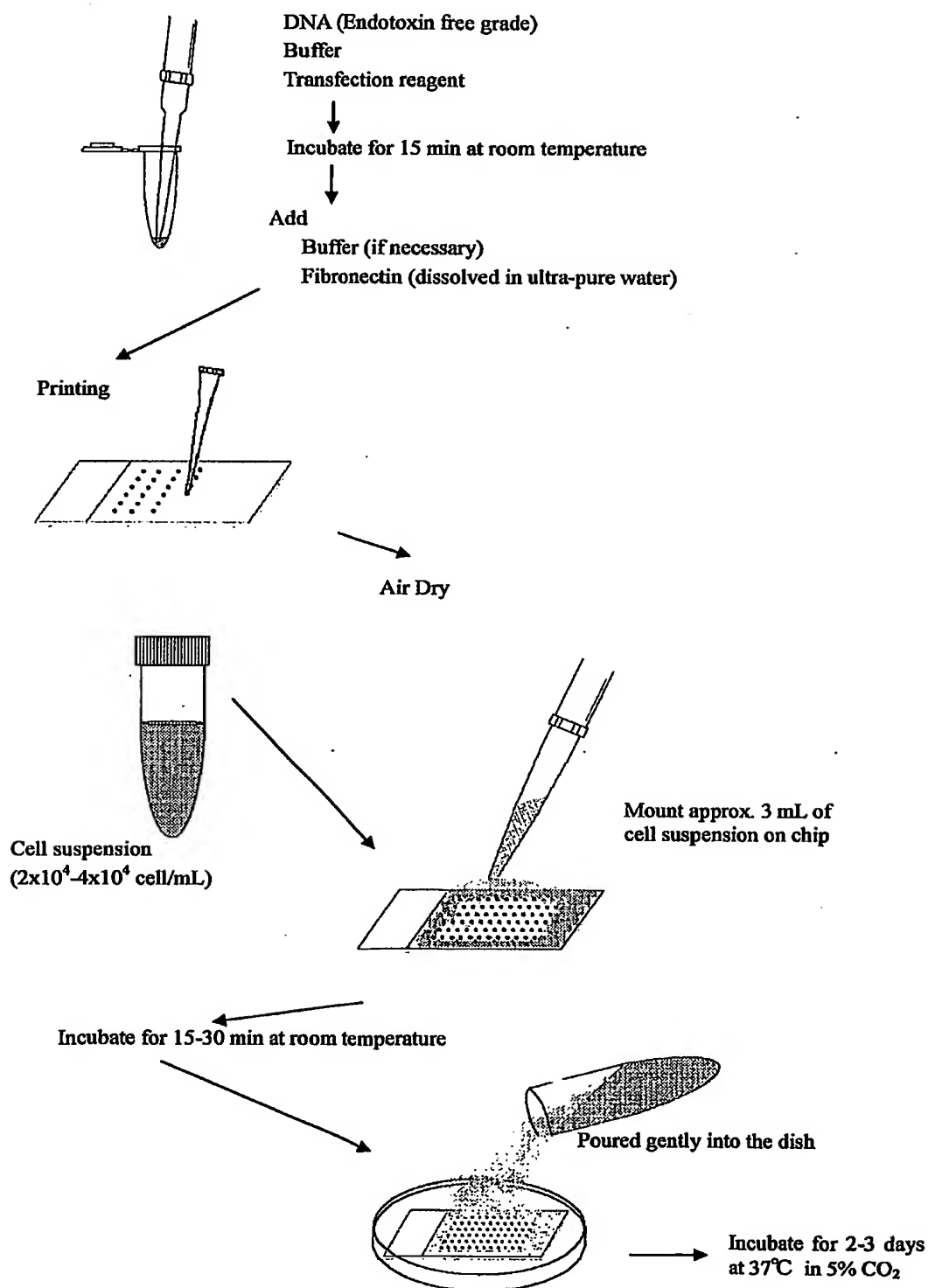
FIG.14C**Solid-Phase Transfection Method**

FIG.14D

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For HEK293

DMEM (serum free)	9.5 uL
Plasmid DNA (1mg/mL)	1.5 uL
TransFast (1mg/mL)	9.0 uL
DMEM (serum free)	5.0 uL
Fibronectin (4mg/mL)	5.0 uL
Final volume	30.0 uL

Scheme for HEK293

1.5mL micro-tube

↓ ←DMEM

↓ ←Plasmid DNA

mix Incubate for 2-3 days
 ↓ ←TransFast at 37°C in 5% CO₂

mix completely and incubate for 15 min at RT

↓ ←DMEM

↓ ←Fibronectin

mix completely

↓

ready to print

For HeLa, NIH3T3-3, HepG2

DMEM (serum free)	14.5 uL
Plasmid DNA (1mg/mL)	1.5 uL
Lipofectamine2000	4.5 uL
DMEM (serum free)	5.0 uL
Fibronectin (4mg/mL)	5.0 uL
Final volume	30.0 uL

Scheme for HeLa, NIH3T3-3, and HepG2

1.5mL micro-tube

↓ ←DMEM

↓ ←Plasmid DNA

mix

↓ ←Lipofectamine2000

mix completely and incubate for 15 min at RT

↓ ←DMEM

↓ ←Fibronectin

mix completely

↓

ready to print

For hMSCs

	N/P=5	N/P=10	N/P=20
DMEM (serum free)	12.75	12.0	10.5 uL
Plasmid DNA (1mg/mL)	1.5	1.5	1.5 uL
JetPBI (x4) conc.	0.75	1.5	3.0 uL
Fibronectin (4mg/mL)	5.0	5.0	5.0 uL
Final volume	20.0	20.0	20.0 uL

Scheme for hMSCs

1.5mL micro-tube

↓ ←DMEM

↓ ←Plasmid DNA

mix

↓ ←jetPBI

mix completely and incubate for 15 min at RT

↓ ←Fibronectin

mix completely

↓

ready to print

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FIG.15A

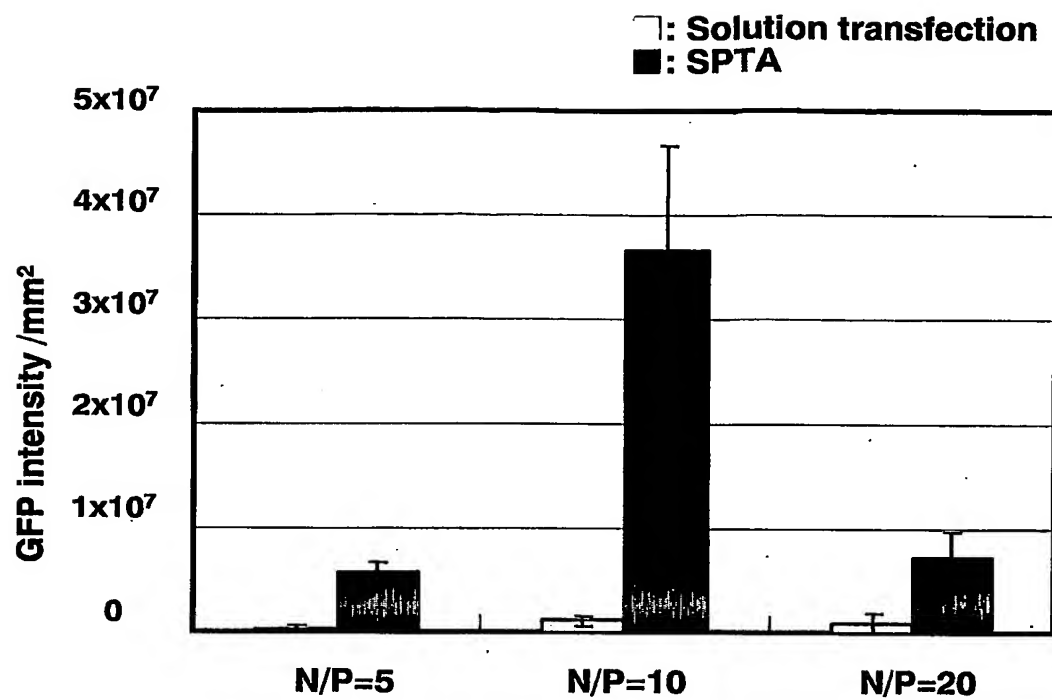


FIG.15B

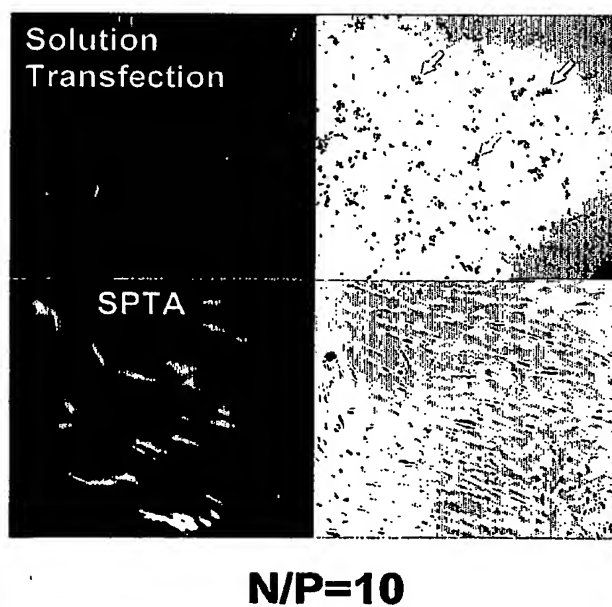
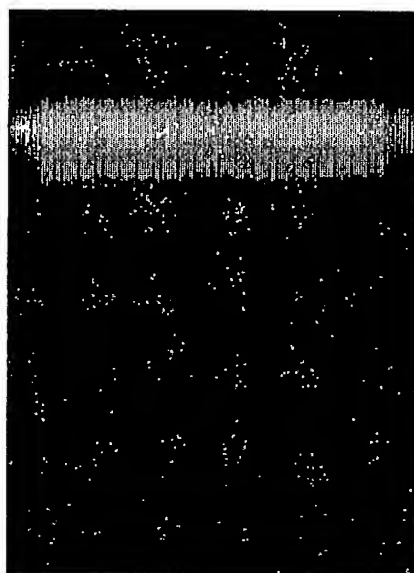


FIG.16B



FIG.16A



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FIG.16C

Number of adherent cells						
	Time(min)					
	0	5	10	15	20	30
APS	235	220	202	157	170	162
APS+gelatin	212	206	184	145	156	183
APS+fibronectin	229	198	183	132	100	85
APS+pronectin L	257	170	126	94	71	47
PLL	231	221	205	162	168	159
PLL+gelatin	218	208	186	151	146	156
PLL+fibronectin	225	174	162	129	98	79
PLL+pronectin L	214	151	132	90	76	50
MAS	231	222	216	182	176	169
MAS+gelatin	224	198	182	163	159	162
MAS+fibronectin	218	182	169	143	112	86
MAS+pronectin L	220	176	152	124	101	66
No coating	226	216	208	192	183	164
Cell adhesion rate (proportion of adherent cells (%))						
	Time(min)					
	0	5	10	15	20	30
APS	0	6.382979	14.04255	33.19149	27.65957	31.06383
APS+gelatin	0	2.830189	13.20755	31.60377	26.41509	13.67925
APS+fibronectin	0	13.53712	20.08734	42.35808	56.33188	62.8821
APS+pronectin L	0	33.85214	50.97276	63.42412	72.37354	81.71206
PLL	0	4.329004	11.25541	29.87013	27.27273	31.16883
PLL+gelatin	0	4.587156	14.6789	30.73394	33.02752	28.44037
PLL+fibronectin	0	22.66667	28	42.66667	56.44444	64.88889
PLL+pronectin L	0	29.43925	38.31776	57.94393	64.48598	76.63551
MAS	0	3.896104	6.493506	21.21212	23.80952	26.83983
MAS+gelatin	0	11.60714	18.75	27.23214	29.01786	27.67857
MAS+fibronectin	0	16.51376	22.47706	34.40367	48.62385	60.55046
MAS+pronectin L	0	20	30.90909	43.63636	54.09091	70
No coating	0	4.424779	7.964602	15.04425	19.02655	27.43363

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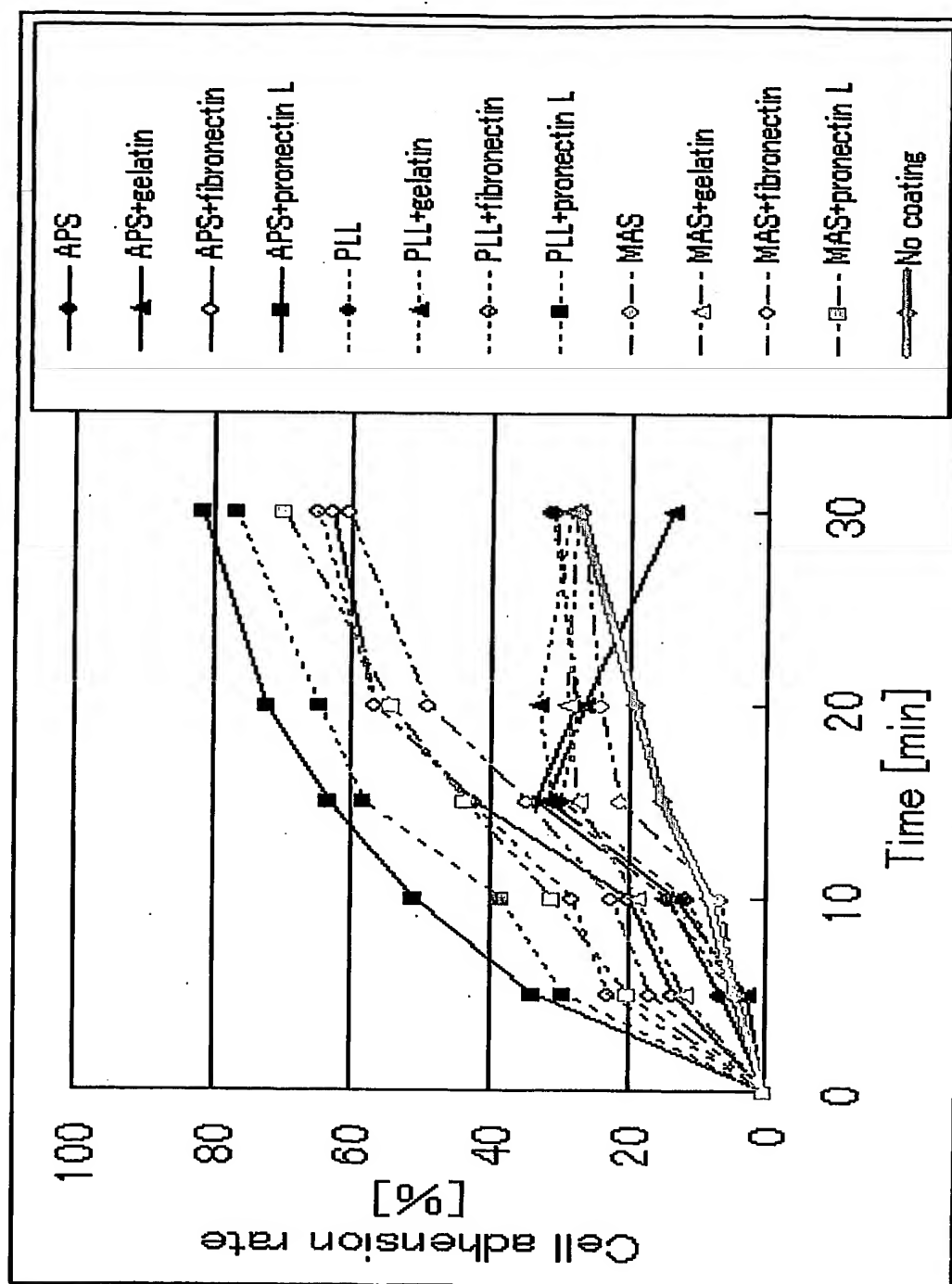
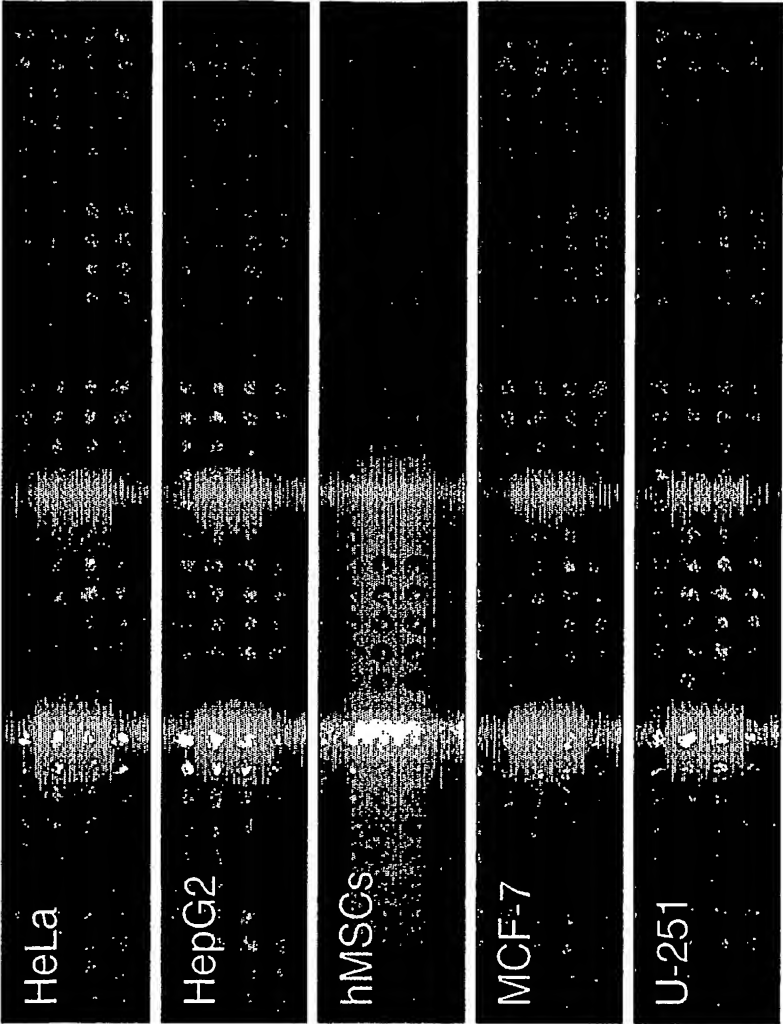
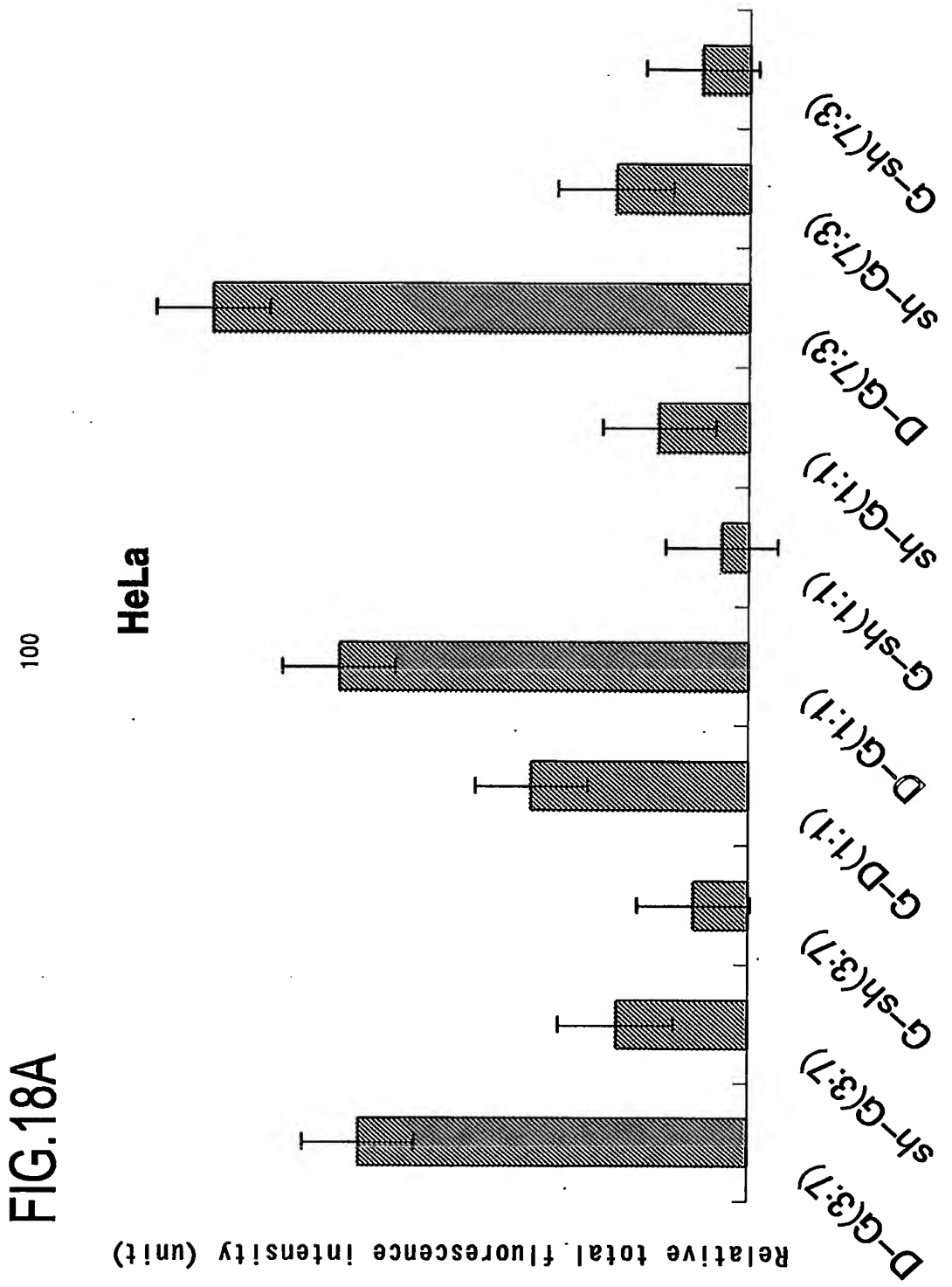


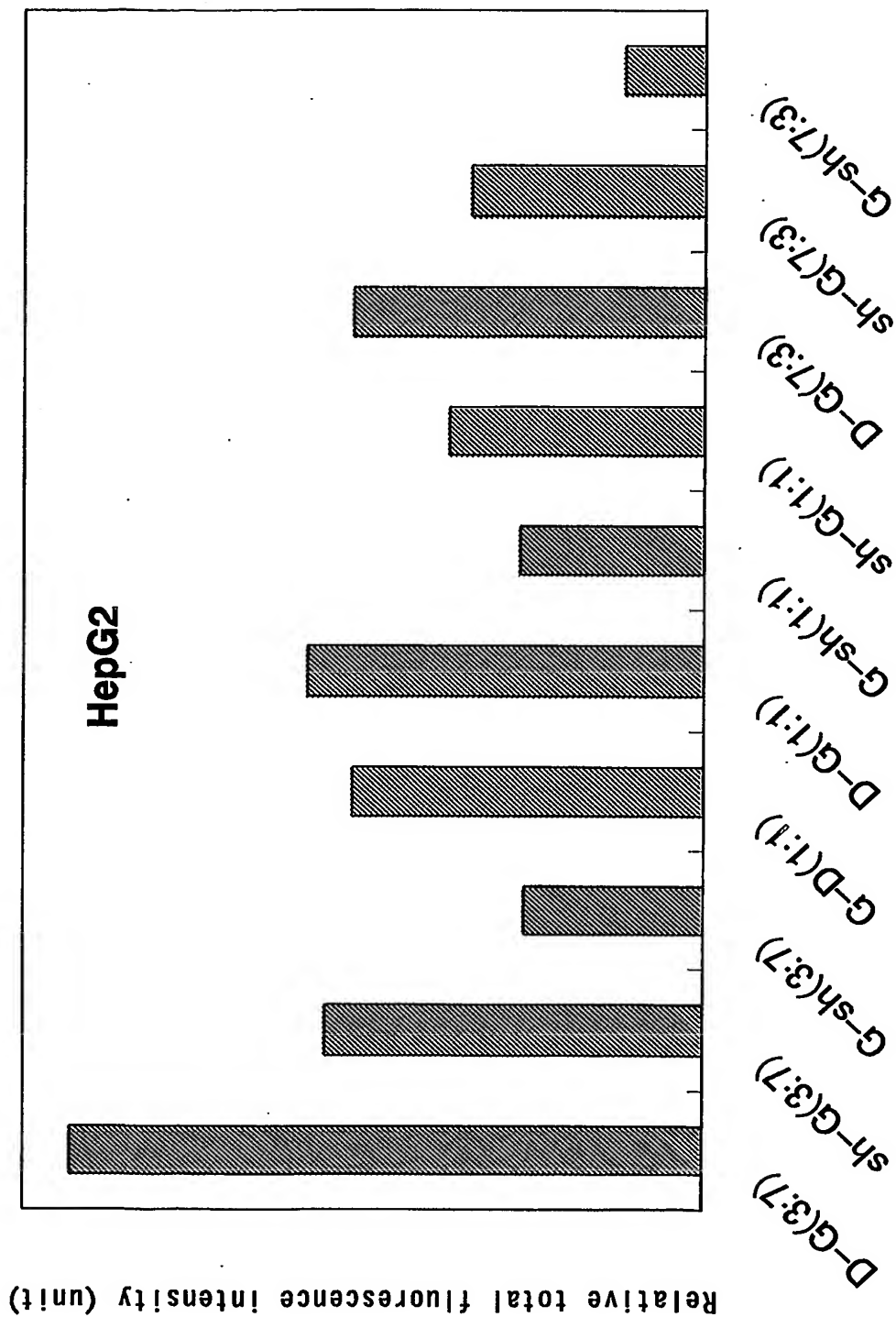
FIG.17





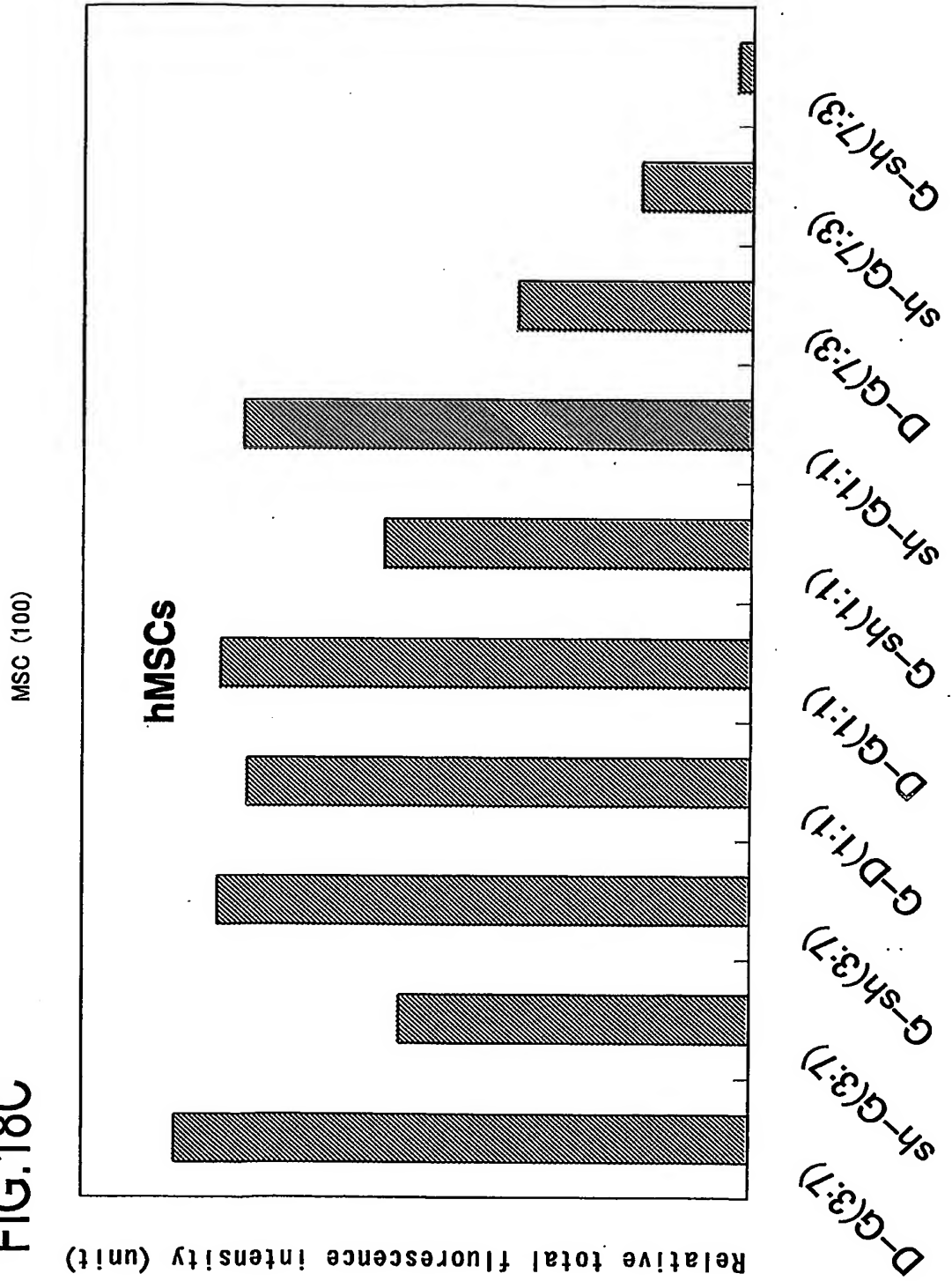
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FIG.18B



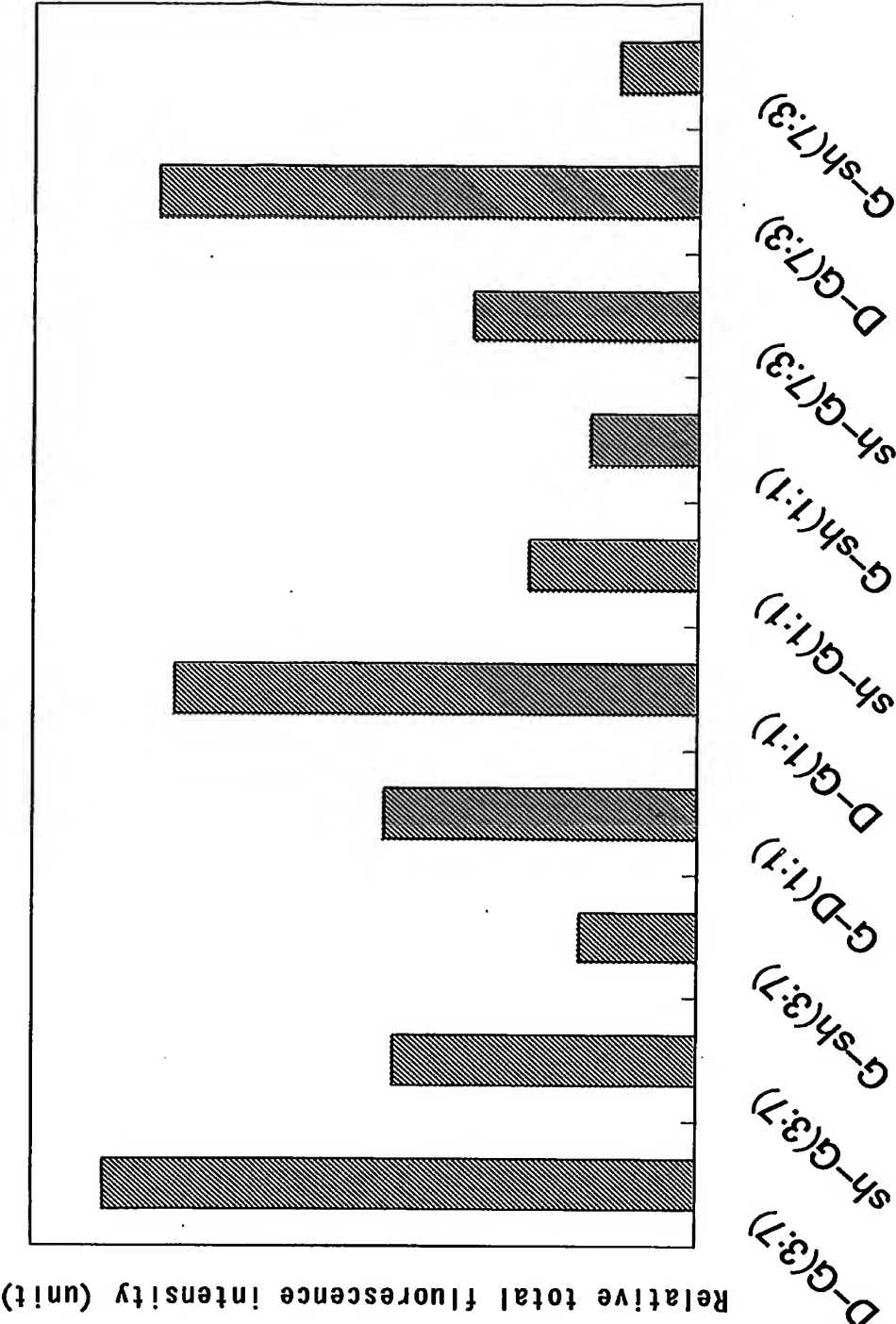
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FIG.18C

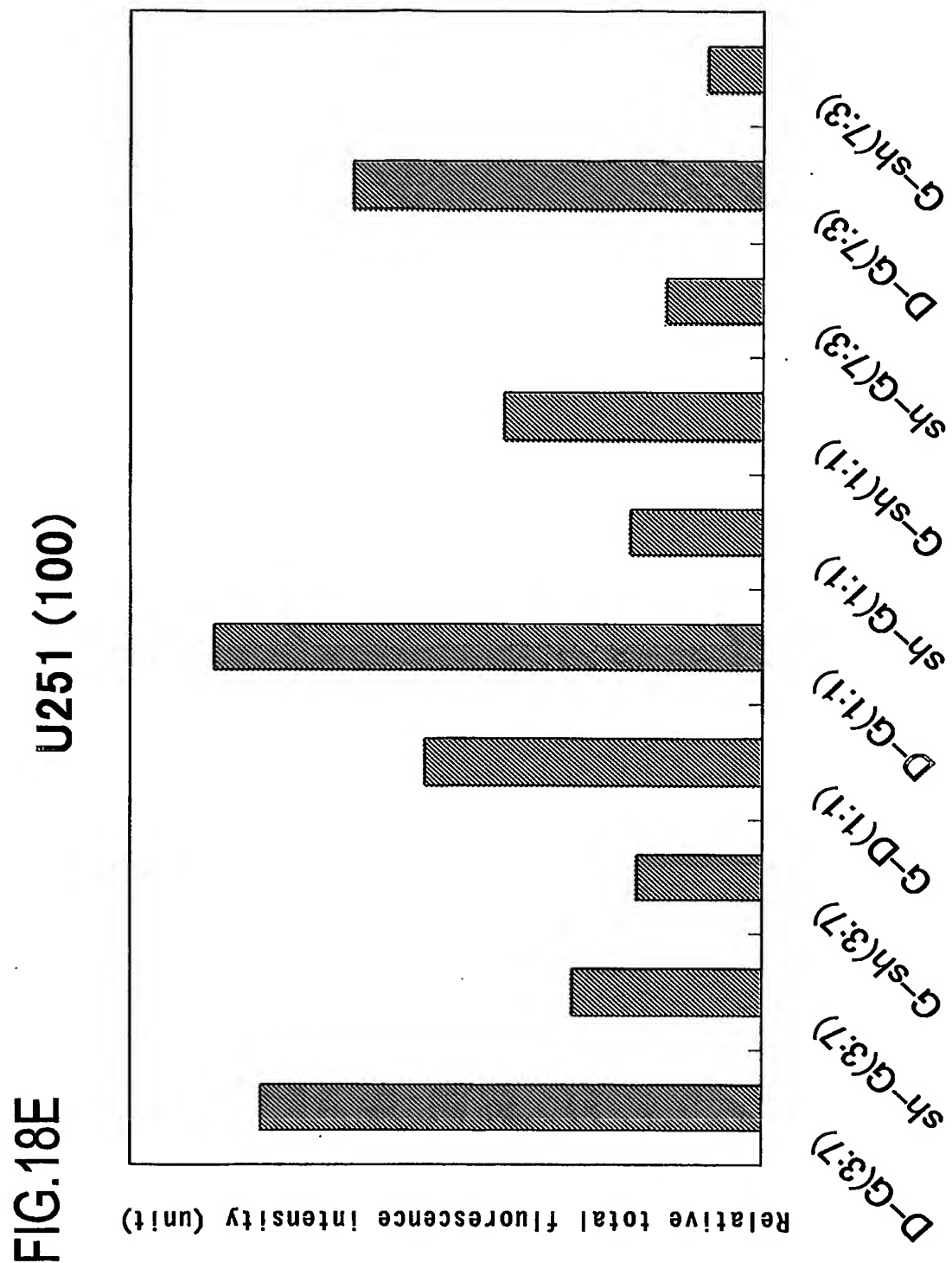


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FIG.18D
MCF7 (100)

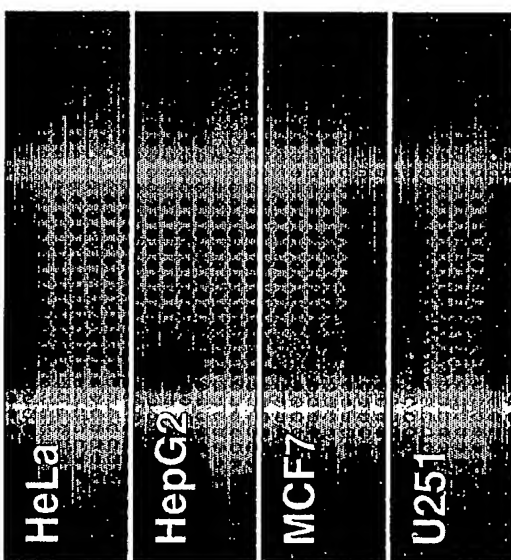


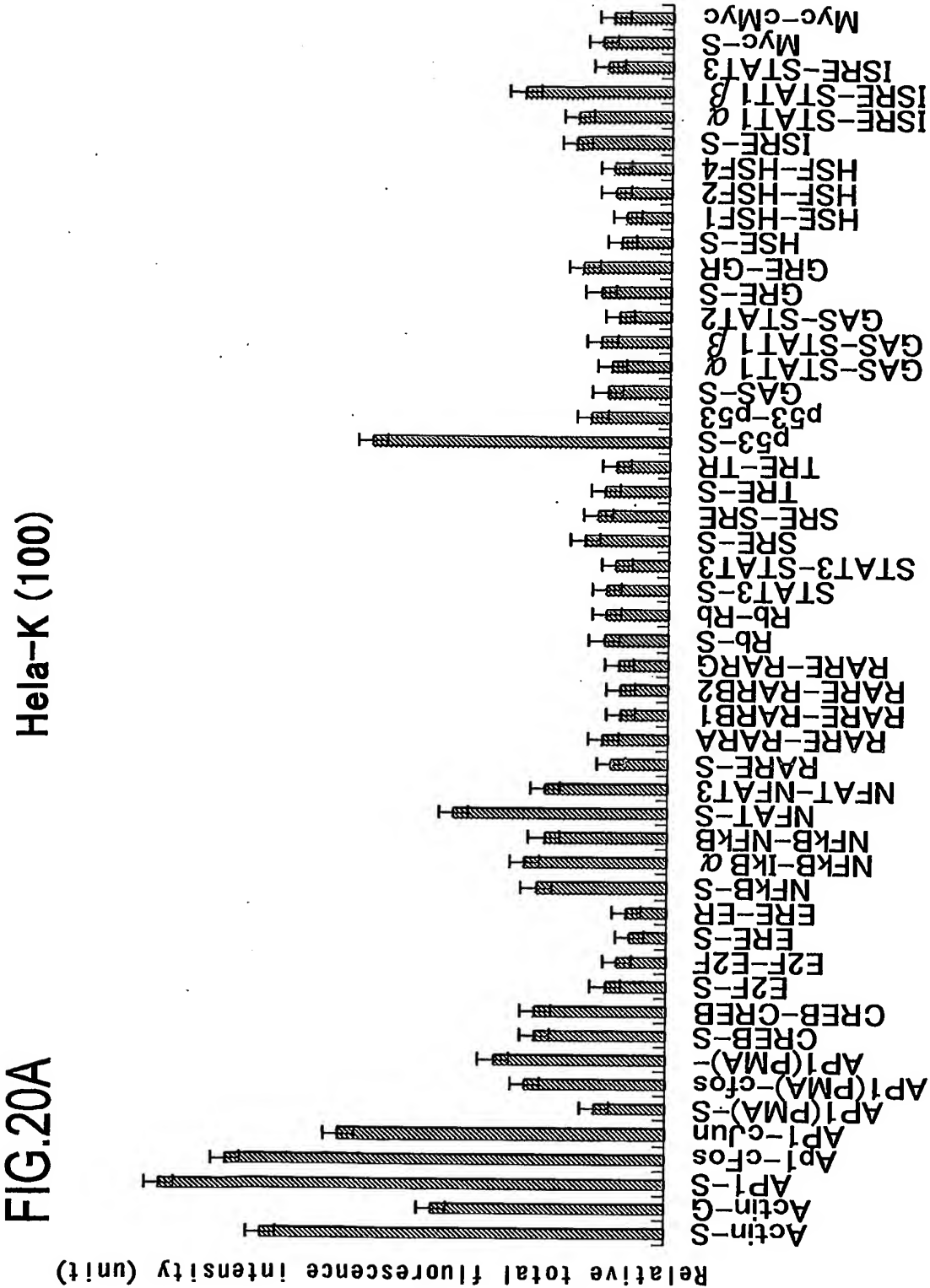
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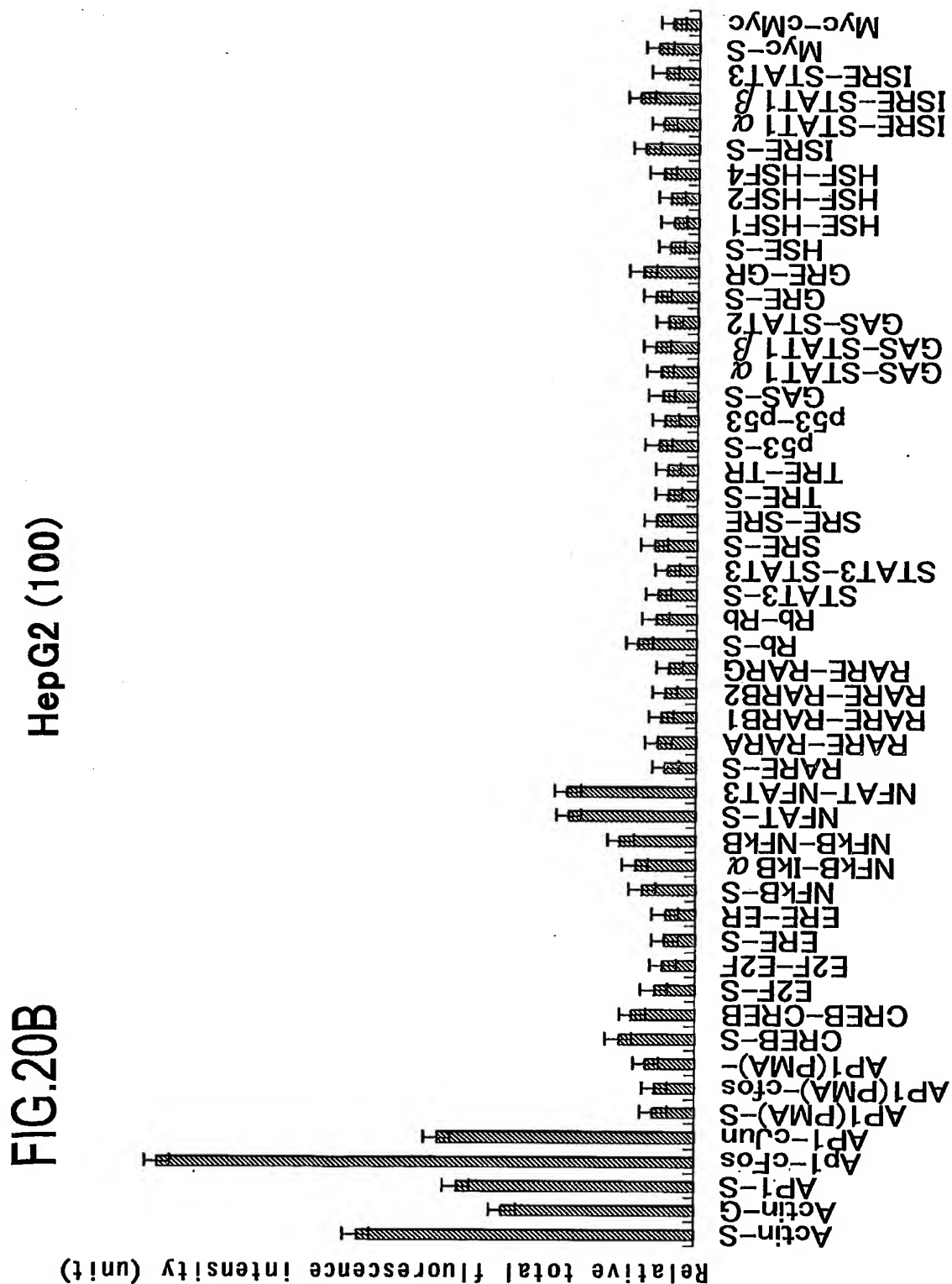
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FIG.19





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MCF7 (100)

FIG.20C

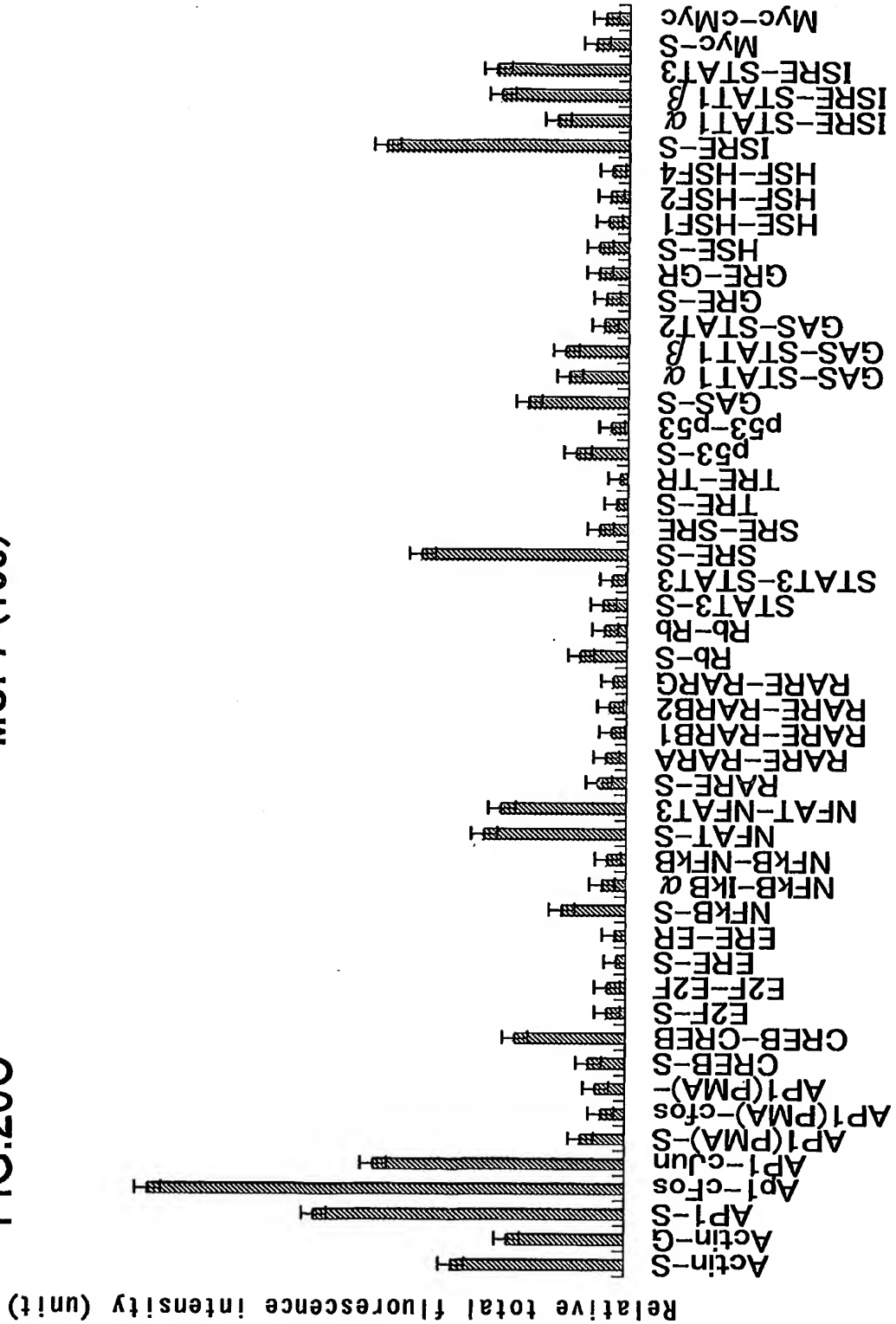
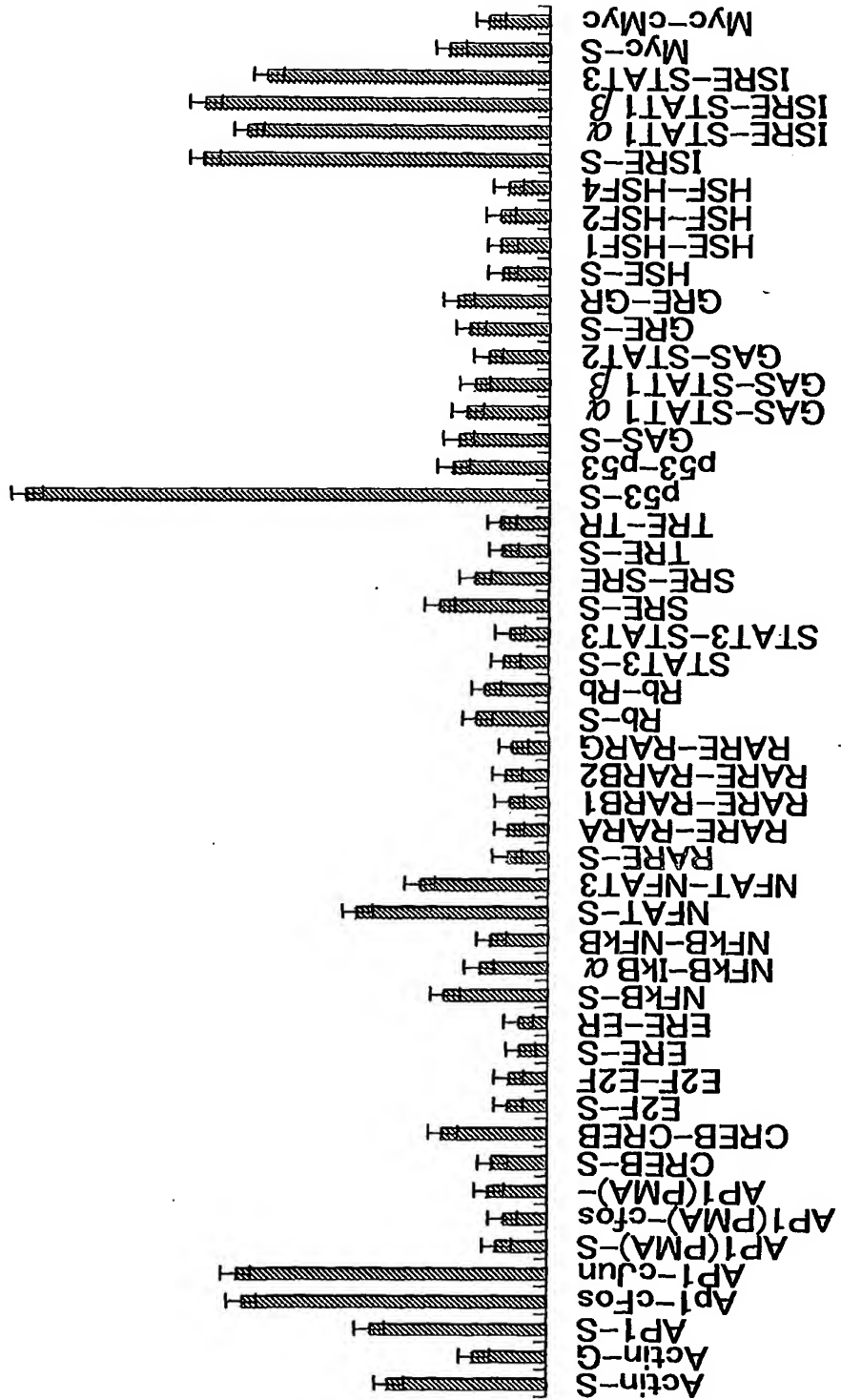


FIG.20D

U251 (100)

Relative total fluorescence intensity (unit)



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FIG.21

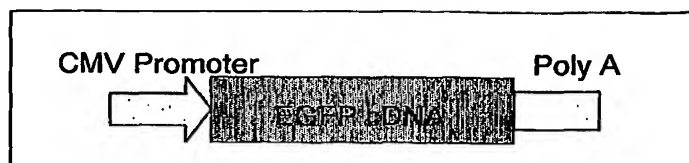


FIG.22

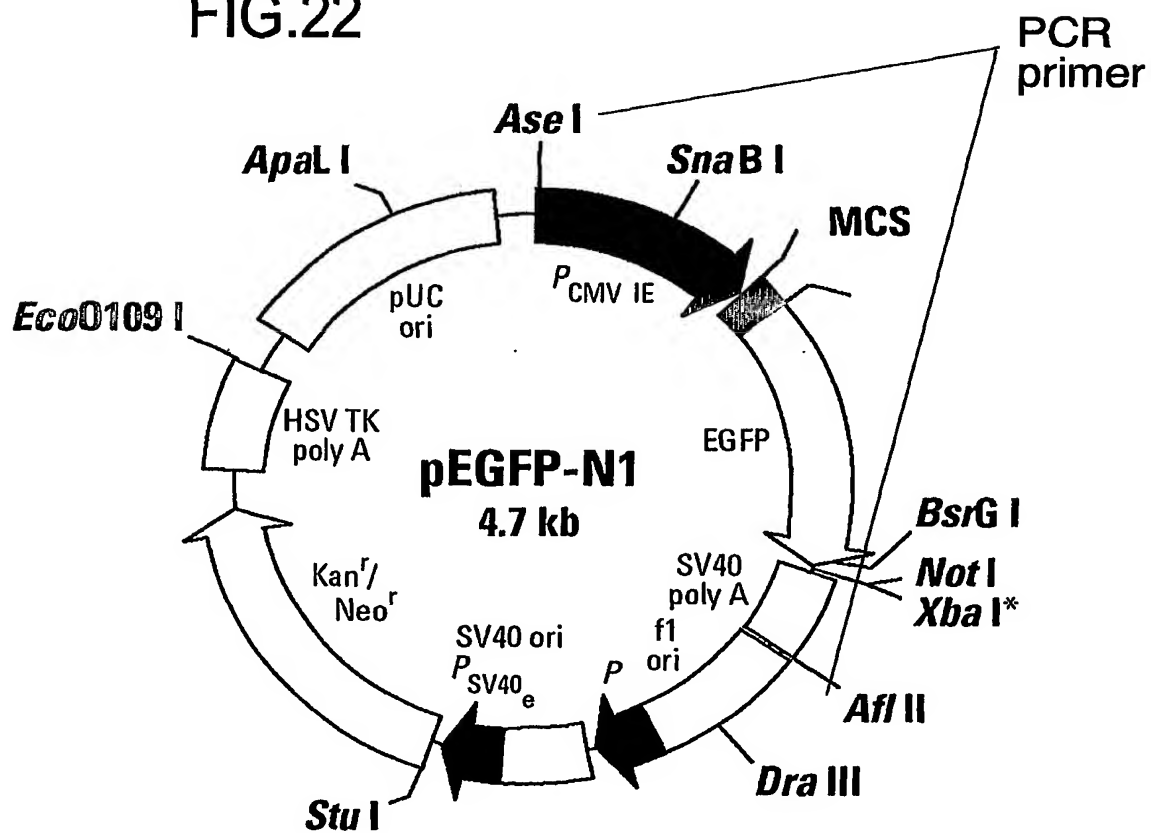


FIG.23

MCF7



Circular DNA

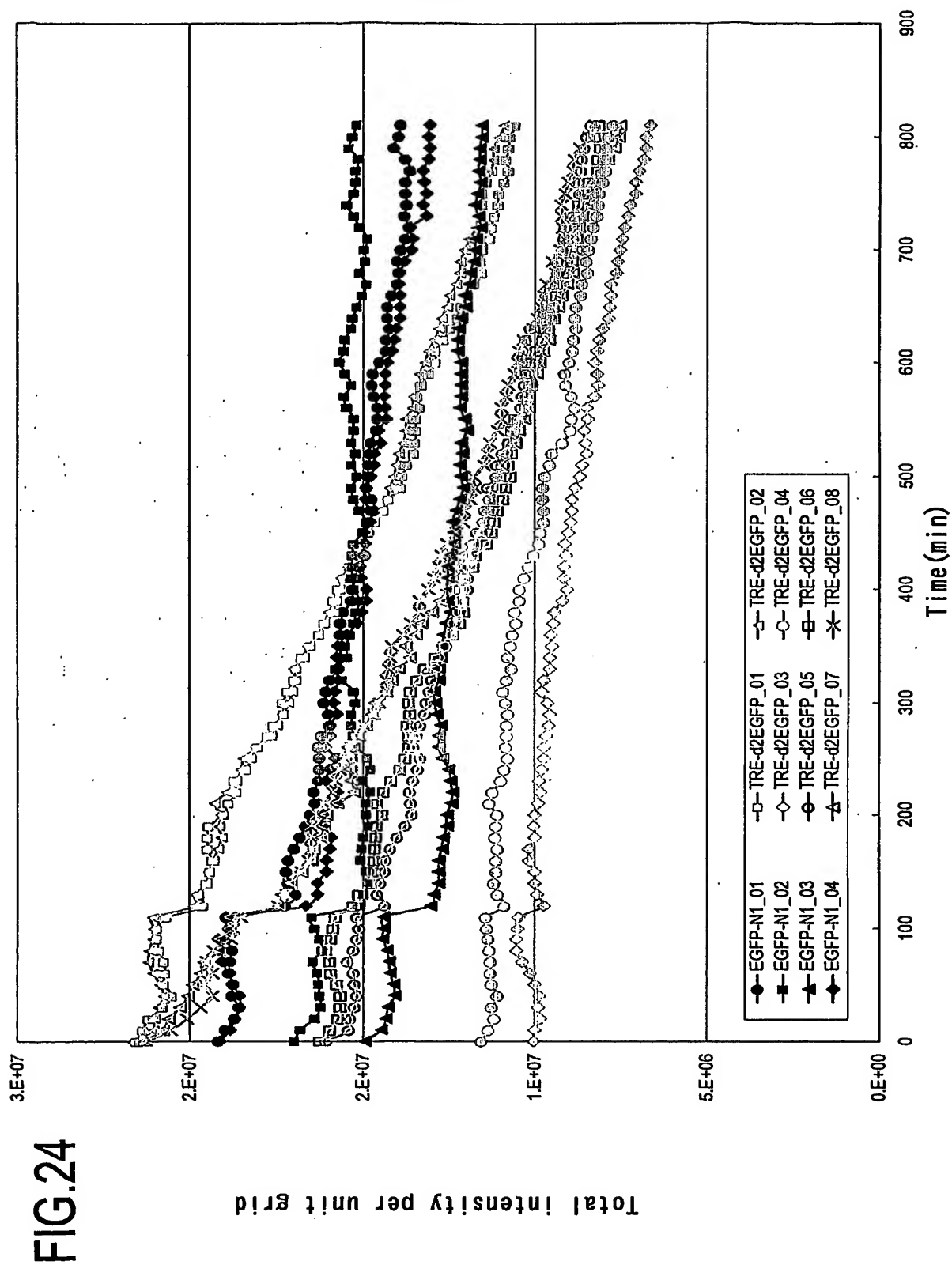
PCR Fragment

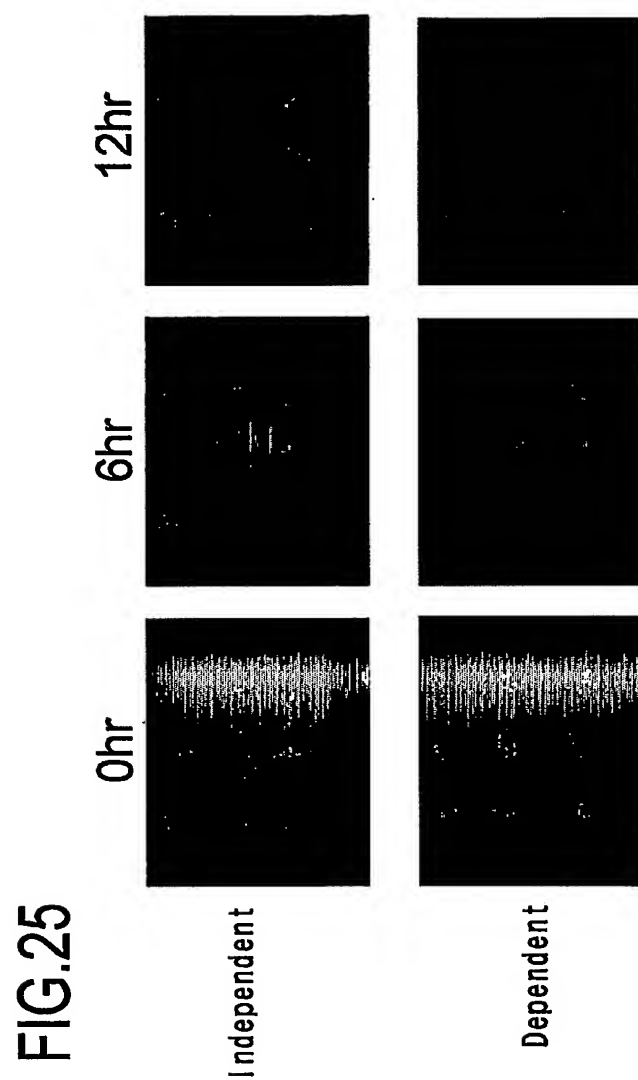
pEGFP-N1

EGFP expression unit

EGFP expression unit

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SEQUENCE LISTING

<110> NATIONAL INSTITUTE OF ADVANCED INDUSTRIAL SCIENCE AND
TECHNOLOGY
MIYAKE, Masato
YOSHIKAWA, Tomohiro
UCHIMURA, Eiichiro
MIYAKE, Jun

<120> COMPOSITION AND METHOD FOR INCREASING EFFICIENCY OF INTRODUCTION OF TARGET
SUBSTANCE INTO CELL

<130> AI003PCT

<150> JP2003-057869

<151> 2003/03/04

<160> 18

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<212> DNA

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<223> fibronectin 1

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Met Leu Arg Gly Pro Gly Pro Gly Leu Leu Leu Leu Ala Val Gln Cys

1 5 10 15

ctg ggg aca gcg gtg ccc tcc acg gga gcc tcg aag agc aag agg cag 96

Leu Gly Thr Ala Val Pro Ser Thr Gly Ala Ser Lys Ser Lys Arg Gln	
20 25 30	
gct cag caa atg gtt cag ccc cag tcc ccg gtg gct gtc agt caa agc	144
Ala Gln Gln Met Val Gln Pro Gln Ser Pro Val Ala Val Ser Gln Ser	
35 40 45	
aag ccc ggt tgt tat gac aat gga aaa cac tat cag ata aat caa cag	192
Lys Pro Gly Cys Tyr Asp Asn Gly Lys His Tyr Gln Ile Asn Gln Gln	
50 55 60	
tgg gag cgg acc tac cta ggc aat gcg ttg gtt tgt act tgt tat gga	240
Trp Glu Arg Thr Tyr Leu Gly Asn Ala Leu Val Cys Thr Cys Tyr Gly	
65 70 75 80	
gga agc cga ggt ttt aac tgc gag agt aaa cct gaa gct gaa gag act	288
Gly Ser Arg Gly Phe Asn Cys Glu Ser Lys Pro Glu Ala Glu Glu Thr	
85 90 95	
tgc ttt gac aag tac act ggg aac act tac cga gtg ggt gac act tat	336
Cys Phe Asp Lys Tyr Thr Gly Asn Thr Tyr Arg Val Gly Asp Thr Tyr	
100 105 110	
gag cgt cct aaa gac tcc atg atc tgg gac tgt acc tgc atc ggg gct	384
Glu Arg Pro Lys Asp Ser Met Ile Trp Asp Cys Thr Cys Ile Gly Ala	
115 120 125	

ggg cga ggg aga ata ago tgt acc atc gca aac cgc tgc cat gaa ggg 432

Gly Arg Gly Arg Ile Ser Cys Thr Ile Ala Asn Arg Cys His Glu Gly

130

135

140

ggt cag tcc tac aag att ggt gac acc tgg agg aga cca cat gag act 480

Gly Gln Ser Tyr Lys Ile Gly Asp Thr Trp Arg Arg Pro His Glu Thr

145

150

155

160

ggt ggt tac atg tta gag tgt gtg tgt ctt ggt aat gga aaa gga gaa 528

Gly Gly Tyr Met Leu Glu Cys Val Cys Leu Gly Asn Gly Lys Gly Glu

165

170

175

tgg acc tgc aag ccc ata gct gag aag tgt ttt gat cat gct gct ggg 576

Trp Thr Cys Lys Pro Ile Ala Glu Lys Cys Phe Asp His Ala Ala Gly

180

185

190

act tcc tat gtg gtc gga gaa acg tgg gag aag ccc tac caa ggc tgg 624

Thr Ser Tyr Val Val Gly Glu Thr Trp Glu Lys Pro Tyr Gln Gly Trp

195

200

205

atg atg gta gat tgt act tgc ctg gga gaa ggc agc gga cgc atc act 672

Met Met Val Asp Cys Thr Cys Leu Gly Glu Gly Ser Gly Arg Ile Thr

210

215

220

tgc act tct aga aat aga tgc aac gat cag gac aca agg aca tcc tat 720

Cys Thr Ser Arg Asn Arg Cys Asn Asp Gln Asp Thr Arg Thr Ser Tyr
 225 230 235 240
 aga att gga gac acc tgg agc aag aag gat aat cga gga aac ctg ctc 768
 Arg Ile Gly Asp Thr Trp Ser Lys Lys Asp Asn Arg Gly Asn Leu Leu
 245 250 255
 cag tgc atc tgc aca ggc aac ggc cga gga gag tgg aag tgt gag agg 816
 Gln Cys Ile Cys Thr Gly Asn Gly Arg Gly Glu Trp Lys Cys Glu Arg
 260 265 270
 cac acc tot gtg cag acc aca tcg agc gga tot ggc ccc ttc acc gat 864
 His Thr Ser Val Gln Thr Thr Ser Ser Gly Ser Gly Pro Phe Thr Asp
 275 280 285
 gtt cgt gca gct gtt tac caa ccg cag cct cac ccc cag cct cct ccc 912
 Val Arg Ala Ala Val Tyr Gln Pro Gln Pro His Pro Gln Pro Pro Pro
 290 295 300
 tat ggc cac tgt gtc aca gac agt ggt gtg gtc tac tct gtg ggg atg 960
 Tyr Gly His Cys Val Thr Asp Ser Gly Val Val Tyr Ser Val Gly Met
 305 310 315 320
 cag tgg ctg aag aca caa gga aat aag caa atg ctt tgc acg tgc ctg 1008
 Gln Trp Leu Lys Thr Gln Gly Asn Lys Gln Met Leu Cys Thr Cys Leu
 325 330 335

ggc aac gga gtc agc tgc caa gag aca gct gta acc cag act tac ggt 1056

Gly Asn Gly Val Ser Cys Gln Glu Thr Ala Val Thr Gln Thr Tyr Gly

340

345

350

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Gly Asn Ser Asn Gly Glu Pro Cys Val Leu Pro Phe Thr Tyr Asn Gly

355

360

365

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Arg Thr Asp Ser Thr Thr Ser Asn Tyr Glu Gln Asp Gln Lys Tyr Ser

370

375

380

ttc tgc aca gac cac act gtt ttg gtt cag act cga gga gga aat tcc 1200

Phe Cys Thr Asp His Thr Val Leu Val Gln Thr Arg Gly Gly Asn Ser

385

390

395

400

aat ggt gcc ttg tgc cac ttc ccc ttc cta tac aac aac cac aat tac 1248

Asn Gly Ala Leu Cys His Phe Pro Phe Leu Tyr Asn Asn His Asn Tyr

405

410

415

act gat tgc act tct gag ggc aga aga gac aac atg aag tgg tgt ggg 1296

Thr Asp Cys Thr Ser Glu Gly Arg Arg Asp Asn Met Lys Trp Cys Gly

420

425

430

acc aca cag aac tat gat gcc gac cag aag ttt ggg ttc tgc ccc atg 1344

Thr Thr Gln Asn Tyr Asp Ala Asp Gln Lys Phe Gly Phe Cys Pro Met
 435 440 445
 gct gcc cac gag gaa atc tgc aca acc aat gaa ggg gtc atg tac cgc 1392
 Ala Ala His Glu Glu Ile Cys Thr Thr Asn Glu Gly Val Met Tyr Arg
 450 455 460
 att gga gat cag tgg gat aag cag cat gac atg ggt cac atg atg agg 1440
 Ile Gly Asp Gln Trp Asp Lys Gln His Asp Met Gly His Met Met Arg
 465 470 475 480
 tgc acg tgt gtt ggg aat ggt cgt ggg gaa tgg aca tgc att gcc tac 1488
 Cys Thr Cys Val Gly Asn Gly Arg Gly Glu Trp Thr Cys Ile Ala Tyr
 485 490 495
 tcg cag ott cga gat cag tgc att gtt gat gac atc act tac aat gtg 1536
 Ser Gln Leu Arg Asp Gln Cys Ile Val Asp Asp Ile Thr Tyr Asn Val
 500 505 510
 aac gac aca ttc cac aag cgt cat gaa gag ggg cac atg ctg aac tgt 1584
 Asn Asp Thr Phe His Lys Arg His Glu Glu Gly His Met Leu Asn Cys
 515 520 525
 aca tgc ttc ggt cag ggt cgg ggc agg tgg aag tgt gat ccc gtc gac 1632
 Thr Cys Phe Gly Gln Gly Arg Gly Arg Trp Lys Cys Asp Pro Val Asp
 530 535 540

caa tgc cag gat tca gag act ggg acg ttt tat caa att gga gat tca 1680
 Gln Cys Gln Asp Ser Glu Thr Gly Thr Phe Tyr Gln Ile Gly Asp Ser
 545 550 555 560

tgg gag aag tat gtg cat ggt gtc aga tac cag tgc tac tgc tat ggc 1728
 Trp Glu Lys Tyr Val His Gly Val Arg Tyr Gln Cys Tyr Cys Tyr Gly
 565 570 575

cgt ggc att ggg gag tgg cat tgc caa cct tta cag acc tat cca agc 1776
 Arg Gly Ile Gly Glu Trp His Cys Gln Pro Leu Gln Thr Tyr Pro Ser
 580 585 590

tca agt ggt cct gtc gaa gta ttt atc act gag act ccg agt cag ccc 1824
 Ser Ser Gly Pro Val Glu Val Phe Ile Thr Glu Thr Pro Ser Gln Pro
 595 600 605

aac tcc cac ccc atc cag tgg aat gca cca cag cca tct cac att tcc 1872
 Asn Ser His Pro Ile Gln Trp Asn Ala Pro Gln Pro Ser His Ile Ser
 610 615 620

aag tac att ctc agg tgg aga cct gtg agt atc cca ccc aga aac ctt 1920
 Lys Tyr Ile Leu Arg Trp Arg Pro Val Ser Ile Pro Pro Arg Asn Leu
 625 630 635 640

gga tac tga 1929

Gly Tyr

<210> 2

<211> 642

<212> PRT

<213> Homo sapiens

<400> 2

Met Leu Arg Gly Pro Gly Pro Gly Leu Leu Leu Leu Ala Val Gln Cys

1 5 10 15

Leu Gly Thr Ala Val Pro Ser Thr Gly Ala Ser Lys Ser Lys Arg Gln

20 25 30

Ala Gln Gln Met Val Gln Pro Gln Ser Pro Val Ala Val Ser Gln Ser

35 40 45

Lys Pro Gly Cys Tyr Asp Asn Gly Lys His Tyr Gln Ile Asn Gln Gln

50 55 60

Trp Glu Arg Thr Tyr Leu Gly Asn Ala Leu Val Cys Thr Cys Tyr Gly
65 70 75 80

Gly Ser Arg Gly Phe Asn Cys Glu Ser Lys Pro Glu Ala Glu Glu Thr

85 90 95

Cys Phe Asp Lys Tyr Thr Gly Asn Thr Tyr Arg Val Gly Asp Thr Tyr
100 105 110

Glu Arg Pro Lys Asp Ser Met Ile Trp Asp Cys Thr Cys Ile Gly Ala

115 120 125

Gly Arg Gly Arg Ile Ser Cys Thr Ile Ala Asn Arg Cys His Glu Gly
130 135 140

Gly Gln Ser Tyr Lys Ile Gly Asp Thr Trp Arg Arg Pro His Glu Thr
145 150 155 160

Gly Gly Tyr Met Leu Glu Cys Val Cys Leu Gly Asn Gly Lys Gly Glu

165

170

175

Trp Thr Cys Lys Pro Ile Ala Glu Lys Cys Phe Asp His Ala Ala Gly

180

185

190

Thr Ser Tyr Val Val Gly Glu Thr Trp Glu Lys Pro Tyr Gln Gly Trp

195

200

205

Met Met Val Asp Cys Thr Cys Leu Gly Glu Gly Ser Gly Arg Ile Thr

210

215

220

Cys Thr Ser Arg Asn Arg Cys Asn Asp Gln Asp Thr Arg Thr Ser Tyr

225

230

235

240

Arg Ile Gly Asp Thr Trp Ser Lys Lys Asp Asn Arg Gly Asn Leu Leu

245

250

255

Gln Cys Ile Cys Thr Gly Asn Gly Arg Gly Glu Trp Lys Cys Glu Arg

260

265

270

His Thr Ser Val Gln Thr Thr Ser Ser Gly Ser Gly Pro Phe Thr Asp
275 280 285

Val Arg Ala Ala Val Tyr Gln Pro Gln Pro His Pro Gln Pro Pro Pro
290 295 300

Tyr Gly His Cys Val Thr Asp Ser Gly Val Val Tyr Ser Val Gly Met
305 310 315 320

Gln Trp Leu Lys Thr Gln Gly Asn Lys Gln Met Leu Cys Thr Cys Leu
325 330 335

Gly Asn Gly Val Ser Cys Gln Glu Thr Ala Val Thr Gln Thr Tyr Gly
340 345 350

Gly Asn Ser Asn Gly Glu Pro Cys Val Leu Pro Phe Thr Tyr Asn Gly
355 360 365

Arg Thr Asp Ser Thr Thr Ser Asn Tyr Glu Gln Asp Gln Lys Tyr Ser

370

375

380

Phe Cys Thr Asp His Thr Val Leu Val Gln Thr Arg Gly Gly Asn Ser

385

390

395

400

Asn Gly Ala Leu Cys His Phe Pro Phe Leu Tyr Asn Asn His Asn Tyr

405

410

415

Thr Asp Cys Thr Ser Glu Gly Arg Arg Asp Asn Met Lys Trp Cys Gly

420

425

430

Thr Thr Gln Asn Tyr Asp Ala Asp Gln Lys Phe Gly Phe Cys Pro Met

435

440

445

Ala Ala His Glu Glu Ile Cys Thr Thr Asn Glu Gly Val Met Tyr Arg

450

455

460

Ile Gly Asp Gln Trp Asp Lys Gln His Asp Met Gly His Met Met Arg

465

470

475

480

Cys Thr Cys Val Gly Asn Gly Arg Gly Glu Trp Thr Cys Ile Ala Tyr
485 490 495

Ser Gln Leu Arg Asp Gln Cys Ile Val Asp Asp Ile Thr Tyr Asn Val
500 505 510

Asn Asp Thr Phe His Lys Arg His Glu Glu Gly His Met Leu Asn Cys
515 520 525

Thr Cys Phe Gly Gln Gly Arg Gly Arg Trp Lys Cys Asp Pro Val Asp
530 535 540

Gln Cys Gln Asp Ser Glu Thr Gly Thr Phe Tyr Gln Ile Gly Asp Ser
545 550 555 560

Trp Glu Lys Tyr Val His Gly Val Arg Tyr Gln Cys Tyr Cys Tyr Gly
565 570 575

Arg Gly Ile Gly Glu Trp His Cys Gln Pro Leu Gln Thr Tyr Pro Ser

580

585

590

Ser Ser Gly Pro Val Glu Val Phe Ile Thr Glu Thr Pro Ser Gln Pro

595

600

605

Asn Ser His Pro Ile Gln Trp Asn Ala Pro Gln Pro Ser His Ile Ser

610

615

620

Lys Tyr Ile Leu Arg Trp Arg Pro Val Ser Ile Pro Pro Arg Asn Leu

625

630

635

640

Gly Tyr

<210> 3

<211> 1437

<212> DNA

<213> *Mus musculus*

<220>

<221> CDS

<222> (1).. (1437)

<223> vitronectin

<400> 3

atg gca ccc ctg agg ccc ttt ttc ata cta gcc ctg gtg gca tgg gtt 48

Met Ala Pro Leu Arg Pro Phe Phe Ile Leu Ala Leu Val Ala Trp Val

1 5 10 15

tct ctg gct gac caa gag tca tgc aag ggc cgc tgc act cag ggt ttc 96

Ser Leu Ala Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe

20 25 30

atg gcc agc aag aag tgt cag tgt gac gag ctt tgc act tac tat cag 144

Met Ala Ser Lys Lys Cys Gln Cys Asp Glu Leu Cys Thr Tyr Tyr Gln

35 40 45

agc tgc tgt gcc gac tac atg gag cag tgc aag ccc caa gta acg cgg 192

Ser Cys Cys Ala Asp Tyr Met Glu Gln Cys Lys Pro Gln Val Thr Arg

50 55 60

ggg gac gtg ttc act atg cca gag gat gat tat tgg agc tat gac tac 240

Gly Asp Val Phe Thr Met Pro Glu Asp Asp Tyr Trp Ser Tyr Asp Tyr

65 70 75 80

gtg gag gag ccc aag aac aat acc aac acc ggt gtg caa ccc gag aac	288
Val Glu Glu Pro Lys Asn Asn Thr Asn Thr Gly Val Gln Pro Glu Asn	
85 90 95	
acc tct cca ccc ggt gac cta aat cct cgg acg gac ggc act cta aag	336
Thr Ser Pro Pro Gly Asp Leu Asn Pro Arg Thr Asp Gly Thr Leu Lys	
100 105 110	
cgg aca gcc ttc cta gat cct gag gaa cag cca agc acc cca gcg cct	384
Pro Thr Ala Phe Leu Asp Pro Glu Glu Gln Pro Ser Thr Pro Ala Pro	
115 120 125	
aaa gtg gag caa cag gag gag atc cta agg ccc gac acc act gat caa	432
Lys Val Glu Gln Gln Glu Glu Ile Leu Arg Pro Asp Thr Thr Asp Gln	
130 135 140	
ggg acc cct gag ttt cca gag gaa gaa ctg tgc agt gga aag ccc ttt	480
Gly Thr Pro Glu Phe Pro Glu Glu Glu Leu Cys Ser Gly Lys Pro Phe	
145 150 155 160	
gac gcc ttc acg gat ctc aag aat ggg tcc ctc ttt gcc ttc cga ggg	528
Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg Gly	
165 170 175	
cag tac cgc tgt gag cta gat gag acg gca gtg agg cct ggg tac ccc	576
Gln Tyr Arg Cys Glu Leu Asp Glu Thr Ala Val Arg Pro Gly Tyr Pro	

180	185	190	
aaa ctt atc caa gat gtc tgg ggc att gag ggc ccc atc gat gct gcc			624
Lys Leu Ile Gln Asp Val Trp Gly Ile Glu Gly Pro Ile Asp Ala Ala			
195	200	205	
ttc act cgc atc aac tgt cag ggg aag acc tac ttg ttc aag ggt agt			672
Phe Thr Arg Ile Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser			
210	215	220	
cag tac tgg cgc ttt gag gat ggg gtc ctg gac cct ggt tat ccc cga			720
Gln Tyr Trp Arg Phe Glu Asp Gly Val Leu Asp Pro Gly Tyr Pro Arg			
225	230	235	240
aac atc tcc gaa ggc ttc agt ggc ata cca gac aat gtt gat gca gcg			768
Asn Ile Ser Glu Gly Phe Ser Gly Ile Pro Asp Asn Val Asp Ala Ala			
245	250	255	
ttc gcc ctt cct gcc cac cgt tac agt ggc cgg gaa agg gtc tac ttc			816
Phe Ala Leu Pro Ala His Arg Tyr Ser Gly Arg Glu Arg Val Tyr Phe			
260	265	270	
ttc aag ggg aag cag tac tgg gag cac gaa ttt cag cag caa ccc ago			864
Phe Lys Gly Lys Gln Tyr Trp Glu His Glu Phe Gln Gln Gln Pro Ser			
275	280	285	

cag gag gag tgc gaa ggc agc tct ctg tca gcc gtg ttt gag cac ttt	912
Gln Glu Glu Cys Glu Gly Ser Ser Leu Ser Ala Val Phe Glu His Phe	
290 295 300	
gcc ttg ctt cag cgg gac agc tgg gag aac att ttc gaa ctc ctc ttc	960
Ala Leu Leu Gln Arg Asp Ser Trp Glu Asn Ile Phe Glu Leu Leu Phe	
305 310 315 320	
tgg ggc aga tcc tct gat gga gcc aga gaa ccc caa ttc atc agc cgg	1008
Trp Gly Arg Ser Ser Asp Gly Ala Arg Glu Pro Gln Phe Ile Ser Arg	
325 330 335	
aac tgg cat ggt gtg cca ggg aaa gtg gac gct gct atg gcc ggc cgc	1056
Asn Trp His Gly Val Pro Gly Lys Val Asp Ala Ala Met Ala Gly Arg	
340 345 350	
atc tac gtc act ggc tcc tta tcc cac tct gcc caa gcc aaa aaa cag	1104
Ile Tyr Val Thr Gly Ser Leu Ser His Ser Ala Gln Ala Lys Lys Gln	
355 360 365	
ccg tct aag cgt aga agc cga aag cgc tat cgt tca cgc cga ggg cgt	1152
Pro Ser Lys Arg Arg Ser Arg Lys Arg Tyr Arg Ser Arg Arg Gly Arg	
370 375 380	
ggc cac aga cgc agc cag ago tcg aac tcc cgt cgt tca tca cgt tca	1200
Gly His Arg Arg Ser Gln Ser Ser Asn Ser Arg Arg Ser Ser Arg Ser	

385 390 395 400
atc tgg ttc tct ttg ttc tcc agc gag gag agt ggg cta gga acc tac 1248
Ile Trp Phe Ser Leu Phe Ser Ser Glu Glu Ser Gly Leu Gly Thr Tyr
405 410 415
aac aac tat gat tat gat atg gac tgg ctt gta cct gcc acc tgc gag 1296
Asn Asn Tyr Asp Tyr Asp Met Asp Trp Leu Val Pro Ala Thr Cys Glu
420 425 430
ccc att cag agc gtc tat ttc ttc tct gga gac aaa tac tac cga gtc 1344
Pro Ile Gln Ser Val Tyr Phe Phe Ser Gly Asp Lys Tyr Tyr Arg Val
435 440 445
aac ctt aga acc cgg cga gtg gac tct gtg aat cct ccc tac cca cgc 1392
Asn Leu Arg Thr Arg Arg Val Asp Ser Val Asn Pro Pro Tyr Pro Arg
450 455 460
tcc att gct cag tat tgg ctg ggc tgc ccg acc tct gag aag tag 1437
Ser Ile Ala Gln Tyr Trp Leu Gly Cys Pro Thr Ser Glu Lys
465 470 475

<210> 4

<211> 478

<212> PRT

<213> Mus musculus

<400> 4

Met Ala Pro Leu Arg Pro Phe Phe Ile Leu Ala Leu Val Ala Trp Val

1 5 10 15

Ser Leu Ala Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe

20 25 30

Met Ala Ser Lys Lys Cys Gln Cys Asp Glu Leu Cys Thr Tyr Tyr Gln

35 40 45

Ser Cys Cys Ala Asp Tyr Met Glu Gln Cys Lys Pro Gln Val Thr Arg

50 55 60

Gly Asp Val Phe Thr Met Pro Glu Asp Asp Tyr Trp Ser Tyr Asp Tyr

65 70 75 80

Val Glu Glu Pro Lys Asn Asn Thr Asn Thr Gly Val Gln Pro Glu Asn

85

90

95

Thr Ser Pro Pro Gly Asp Leu Asn Pro Arg Thr Asp Gly Thr Leu Lys

100

105

110

Pro Thr Ala Phe Leu Asp Pro Glu Glu Gln Pro Ser Thr Pro Ala Pro

115

120

125

Lys Val Glu Gln Gln Glu Glu Ile Leu Arg Pro Asp Thr Thr Asp Gln

130

135

140

Gly Thr Pro Glu Phe Pro Glu Glu Glu Leu Cys Ser Gly Lys Pro Phe

145

150

155

160

Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg Gly

165

170

175

Gln Tyr Arg Cys Glu Leu Asp Glu Thr Ala Val Arg Pro Gly Tyr Pro

180

185

190

Lys Leu Ile Gln Asp Val Trp Gly Ile Glu Gly Pro Ile Asp Ala Ala

195

200

205

Phe Thr Arg Ile Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser

210

215

220

Gln Tyr Trp Arg Phe Glu Asp Gly Val Leu Asp Pro Gly Tyr Pro Arg

225

230

235

240

Asn Ile Ser Glu Gly Phe Ser Gly Ile Pro Asp Asn Val Asp Ala Ala

245

250

255

Phe Ala Leu Pro Ala His Arg Tyr Ser Gly Arg Glu Arg Val Tyr Phe

260

265

270

Phe Lys Gly Lys Gln Tyr Trp Glu His Glu Phe Gln Gln Gln Pro Ser

275

280

285

Gln Glu Glu Cys Glu Gly Ser Ser Leu Ser Ala Val Phe Glu His Phe

290

295

300

Ala Leu Leu Gln Arg Asp Ser Trp Glu Asn Ile Phe Glu Leu Leu Phe

305

310

315

320

Trp Gly Arg Ser Ser Asp Gly Ala Arg Glu Pro Gln Phe Ile Ser Arg

325

330

335

Asn Trp His Gly Val Pro Gly Lys Val Asp Ala Ala Met Ala Gly Arg

340

345

350

Ile Tyr Val Thr Gly Ser Leu Ser His Ser Ala Gln Ala Lys Lys Gln

355

360

365

Pro Ser Lys Arg Arg Ser Arg Lys Arg Tyr Arg Ser Arg Arg Gly Arg

370

375

380

Gly His Arg Arg Ser Gln Ser Ser Asn Ser Arg Arg Ser Ser Arg Ser

385

390

395

400

Ile Trp Phe Ser Leu Phe Ser Ser Glu Glu Ser Gly Leu Gly Thr Tyr

405

410

415

Asn Asn Tyr Asp Tyr Asp Met Asp Trp Leu Val Pro Ala Thr Cys Glu

420

425

430

Pro Ile Gln Ser Val Tyr Phe Phe Ser Gly Asp Lys Tyr Tyr Arg Val

435

440

445

Asn Leu Arg Thr Arg Arg Val Asp Ser Val Asn Pro Pro Tyr Pro Arg

450

455

460

Ser Ile Ala Gln Tyr Trp Leu Gly Cys Pro Thr Ser Glu Lys

465

470

475

<210> 5

<211> 9511

<212> DNA

<213> Mus musculus

<220>

<221> CDS

<222> (121).. (9372)

<223> laminin-2 alpha chain

<400> 5

ggcacgagct gcaactccgt gggotccggg aggagtggat ctgctcoggc caggatgcct 60

gcggccaccg cggggatcct ctigctcctg ctottgggga cgtcgaagg ctcccagact 120

cag cgg cga cag tcc caa gcg cat caa cag aga ggt tta ttt cct gct 168

Gln Arg Arg Gln Ser Gln Ala His Gln Gln Arg Gly Leu Phe Pro Ala

1 5 10 15

gtc ctg aat ctt gct tgc aat gca ctc atc aca acc aat gct aca tgt 216

Val Leu Asn Leu Ala Ser Asn Ala Leu Ile Thr Thr Asn Ala Thr Cys

20 25 30

ggg gaa aaa gga ccc gag atg tac tgc aag ttg gtg gaa cat gtc ccc 264

Gly Glu Lys Gly Pro Glu Met Tyr Cys Lys Leu Val Glu His Val Pro

35 40 45

ggg cag cct gtg agg aac cct cag tgc cga atc tgc aat cag aac agc 312

Gly Gln Pro Val Arg Asn Pro Gln Cys Arg Ile Cys Asn Gln Asn Ser

50	55	60	
agc aat cca tac cag agg cac ccg att acg aat got att gat ggc aag			360
Ser Asn Pro Tyr Gln Arg His Pro Ile Thr Asn Ala Ile Asp Gly Lys			
65	70	75	80
aac aca tgg tgg cag agt ccc agt atc aag aat gga gtg gaa tac cat			408
Asn Thr Trp Trp Gln Ser Pro Ser Ile Lys Asn Gly Val Glu Tyr His			
	85	90	95
tat gtg aca att act ctg gat tta cag cag gtg ttc cag att gcc tac			456
Tyr Val Thr Ile Thr Leu Asp Leu Gln Gln Val Phe Gln Ile Ala Tyr			
	100	105	110
gta att gtg aag gca gcc aat tcc cct cgg cct gga aac tgg att ttg			504
Val Ile Val Lys Ala Ala Asn Ser Pro Arg Pro Gly Asn Trp Ile Leu			
	115	120	125
gaa cgt tcc ctg gat gac gtg gag tac aaa ccc tgg cag tat cat gcg			552
Glu Arg Ser Leu Asp Asp Val Glu Tyr Lys Pro Trp Gln Tyr His Ala			
	130	135	140
gtg aca gac acg gag tgc ctg acc ctc tac aat atc tat ccc cgc act			600
Val Thr Asp Thr Glu Cys Leu Thr Leu Tyr Asn Ile Tyr Pro Arg Thr			
145	150	155	160

gga cca cca tcc tac gcc aaa gat gat gag gtc atc tgc act tca ttt 648

Gly Pro Pro Ser Tyr Ala Lys Asp Asp Glu Val Ile Cys Thr Ser Phe

165

170

175

tat tcg aag atc cac cct tta gaa aat gga gag att cac att tot ttg 696

Tyr Ser Lys Ile His Pro Leu Glu Asn Gly Glu Ile His Ile Ser Leu

180

185

190

atc aat ggg aga cca agt gct gat gac ccc tcc cct gaa ctc ctg gaa 744

Ile Asn Gly Arg Pro Ser Ala Asp Asp Pro Ser Pro Glu Leu Leu Glu

195

200

205

ttc acc tot gct cgc tac att cgc ctg aga ttt cag agg atc cgc acc 792

Phe Thr Ser Ala Arg Tyr Ile Arg Leu Arg Phe Gln Arg Ile Arg Thr

210

215

220

ttg aat gca gac ttg atg atg ttt gct cac aaa gac ccc aga gaa atc 840

Leu Asn Ala Asp Leu Met Met Phe Ala His Lys Asp Pro Arg Glu Ile

225

230

235

240

gat ccc att gtc aca cga aga tat tac tat tct gtc aag gat att tca 888

Asp Pro Ile Val Thr Arg Arg Tyr Tyr Tyr Ser Val Lys Asp Ile Ser

245

250

255

gtt ggc ggg atg tgc atc tgt tat ggt cat gcc cgg gct tgt cca ctt 936

Val Gly Gly Met Cys Ile Cys Tyr Gly His Ala Arg Ala Cys Pro Leu

260	265	270	
gac cct gca aca aat aaa tca cgc tgt gag tgt gaa cat aac acc tgt			984
Asp Pro Ala Thr Asn Lys Ser Arg Cys Glu Cys Glu His Asn Thr Cys			
275	280	285	
ggg gaa agc tgt gac agg tgc tgt cca gga ttc cat cag aag cct tgg			1032
Gly Glu Ser Cys Asp Arg Cys Cys Pro Gly Phe His Gln Lys Pro Trp			
290	295	300	
aga gct gga acc ttc ctc acc aag tot gag tgt gaa gca tgc aat tgt			1080
Arg Ala Gly Thr Phe Leu Thr Lys Ser Glu Cys Glu Ala Cys Asn Cys			
305	310	315	320
cac gga aaa gct gag gaa tgc tat tat gat gaa act gtt gct agc aga			1128
His Gly Lys Ala Glu Glu Cys Tyr Tyr Asp Glu Thr Val Ala Ser Arg			
325	330	335	
aat cta agt tta aat ata cat ggg aag tac atc gga ggg ggt gtg tgc			1176
Asn Leu Ser Leu Asn Ile His Gly Lys Tyr Ile Gly Gly Gly Val Cys			
340	345	350	
atc aac tgc aca cat aac acg gct ggg ata aat tgt gag aca tgt gtt			1224
Ile Asn Cys Thr His Asn Thr Ala Gly Ile Asn Cys Glu Thr Cys Val			
355	360	365	

gat gga ttc ttc aga ccc aaa ggg gtg tca cca aat tat cca aga cca	1272
Asp Gly Phe Phe Arg Pro Lys Gly Val Ser Pro Asn Tyr Pro Arg Pro	
370 375 380	
tgc cag cca tgt cac tgt gat cca act ggc tcc ott agt gaa gtc tgt	1320
Cys Gln Pro Cys His Cys Asp Pro Thr Gly Ser Leu Ser Glu Val Cys	
385 390 395 400	
gtc aaa gat gag aaa tac gcc cag cga ggg ttg aaa cct gga tcc tgt	1368
Val Lys Asp Glu Lys Tyr Ala Gln Arg Gly Leu Lys Pro Gly Ser Cys	
405 410 415	
cac tgc aaa act ggc ttt gga ggc gtg aac tgt gat cgc tgt gtc agg	1416
His Cys Lys Thr Gly Phe Gly Gly Val Asn Cys Asp Arg Cys Val Arg	
420 425 430	
ggt tac cat ggt tac cca gac tgc caa ccc tgt aac tgt agt ggc ttg	1464
Gly Tyr His Gly Tyr Pro Asp Cys Gln Pro Cys Asn Cys Ser Gly Leu	
435 440 445	
ggg agc aca aat gag gac cct tgc gtt ggg ccc tgt ago tgt aag gag	1512
Gly Ser Thr Asn Glu Asp Pro Cys Val Gly Pro Cys Ser Cys Lys Glu	
450 455 460	
aat gtt gaa ggt gaa gac tgt agt cgt tgc aaa tct ggt ttc ttc aac	1560
Asn Val Glu Gly Glu Asp Cys Ser Arg Cys Lys Ser Gly Phe Phe Asn	

465	470	475	480	
ttg caa gaa gat aat cag aaa ggc tgt gag gag tgt ttc tgt tca gga				1608
Leu Gln Glu Asp Asn Gln Lys Gly Cys Glu Glu Cys Phe Cys Ser Gly				
	485	490	495	
gta tca aac aga tgt cag agt tcc tac tgg acc tat ggg aat att caa				1656
Val Ser Asn Arg Cys Gln Ser Ser Tyr Trp Thr Tyr Gly Asn Ile Gln				
	500	505	510	
gac atg cgt ggt tgg tat ctc aca gac ctc tct ggc cgc att cgg atg				1704
Asp Met Arg Gly Trp Tyr Leu Thr Asp Leu Ser Gly Arg Ile Arg Met				
	515	520	525	
gct ccc cag ctt gat aac cct gac tca cct cag cag atc agc atc agt				1752
Ala Pro Gln Leu Asp Asn Pro Asp Ser Pro Gln Gln Ile Ser Ile Ser				
	530	535	540	
aac tct gag gcc cgg aaa tcc ctg ctt gat ggt tac tac tgg agt gca				1800
Asn Ser Glu Ala Arg Lys Ser Leu Leu Asp Gly Tyr Tyr Trp Ser Ala				
545	550	555	560	
ccg cct cca tat ctg gga aac aga ctt cca gct gtt ggg gga cag ttg				1848
Pro Pro Pro Tyr Leu Gly Asn Arg Leu Pro Ala Val Gly Gly Gln Leu				
	565	570	575	

tca ttt acc atc tca tat gac ctc gaa gaa gag gaa gac gat aca gaa 1896
Ser Phe Thr Ile Ser Tyr Asp Leu Glu Glu Glu Glu Asp Asp Thr Glu
580 585 590

aaa ctc ctt cag ctg atg att atc ttt gag gga aat gac tta aga atc 1944
Lys Leu Leu Gln Leu Met Ile Ile Phe Glu Gly Asn Asp Leu Arg Ile
595 600 605

agc aca gcg tat aag gag gtg tac tta gag cca tct gaa gaa cac gtt 1992
Ser Thr Ala Tyr Lys Glu Val Tyr Leu Glu Pro Ser Glu Glu His Val
610 615 620

gag gag gtg tca ctc aaa gaa gag gcc ttt act ata cat gga aca aat 2040
Glu Glu Val Ser Leu Lys Glu Glu Ala Phe Thr Ile His Gly Thr Asn
625 630 635 640

ttg cca gtc act aga aaa gat ttc atg att gtt ctc aca aat ttg gga 2088
Leu Pro Val Thr Arg Lys Asp Phe Met Ile Val Leu Thr Asn Leu Gly
645 650 655

gag atc ctt atc caa atc aca tac aac tta ggg atg gac gcc atc ttc 2136
Glu Ile Leu Ile Gln Ile Thr Tyr Asn Leu Gly Met Asp Ala Ile Phe
660 665 670

agg ctg agt tct gtc aat ctt gaa tct cct gtc cct tat cct act gat 2184
Arg Leu Ser Ser Val Asn Leu Glu Ser Pro Val Pro Tyr Pro Thr Asp

675	680	685	
aga cgt att gca act gat gtg gaa gtt tgc cag tgt cca cct ggg tac			2232
Arg Arg Ile Ala Thr Asp Val Glu Val Cys Gln Cys Pro Pro Gly Tyr			
690	695	700	
agt ggc agc tct tgt gaa aca tgt tgg cct agg cac cga aga gtt aac			2280
Ser Gly Ser Ser Cys Glu Thr Cys Trp Pro Arg His Arg Arg Val Asn			
705	710	715	720
ggc acc att ttt ggt ggc att tgt gaa cca tgt cag tgc ttt gct cat			2328
Gly Thr Ile Phe Gly Gly Ile Cys Glu Pro Cys Gln Cys Phe Ala His			
725	730	735	
gca gaa gcc tgt gat gac atc aca gga gaa tgt ctg aac tgt aag gat			2376
Ala Glu Ala Cys Asp Asp Ile Thr Gly Glu Cys Leu Asn Cys Lys Asp			
740	745	750	
cac aca ggt ggg cgg tac tgc aat gaa tgt ctc cct gga ttc tat ggt			2424
His Thr Gly Gly Pro Tyr Cys Asn Glu Cys Leu Pro Gly Phe Tyr Gly			
755	760	765	
gat cct act cga gga agc cct gaa gac tgt cag ccc tgt gcc tgt cca			2472
Asp Pro Thr Arg Gly Ser Pro Glu Asp Cys Gln Pro Cys Ala Cys Pro			
770	775	780	

ctc aat atc cca tca aat aac ttt agt cca aca tgc cat tta gac cgg	2520
Leu Asn Ile Pro Ser Asn Asn Phe Ser Pro Thr Cys His Leu Asp Arg	
785 790 795 800	
agt ctg gga ttg atc tgt gac gag tgt cct att ggg tac aca gga ccg	2568
Ser Leu Gly Leu Ile Cys Asp Glu Cys Pro Ile Gly Tyr Thr Gly Pro	
805 810 815	
cgc tgt gag agg tgt gca gaa ggc tat ttt gga caa cct tcc gta cct	2616
Arg Cys Glu Arg Cys Ala Glu Gly Tyr Phe Gly Gln Pro Ser Val Pro	
820 825 830	
gga gga tca tgt cag cca tgc caa tgc aat gac aac ctt gac tac tcc	2664
Gly Gly Ser Cys Gln Pro Cys Gln Cys Asn Asp Asn Leu Asp Tyr Ser	
835 840 845	
atc cct ggc agc tgt gac agc ctg tct ggc tcc tgt ctg att tgt aag	2712
Ile Pro Gly Ser Cys Asp Ser Leu Ser Gly Ser Cys Leu Ile Cys Lys	
850 855 860	
cca ggt aca aca ggc cgg tac tgt gag ctc tgt gct gat ggg tat ttt	2760
Pro Gly Thr Thr Gly Arg Tyr Cys Glu Leu Cys Ala Asp Gly Tyr Phe	
865 870 875 880	
gga gac gcg gtt aat aca aag aac tgt caa cca tgc cgt tgt gat atc	2808
Gly Asp Ala Val Asn Thr Lys Asn Cys Gln Pro Cys Arg Cys Asp Ile	

885	890	895	
aat ggc tcc ttc tca gag gat tgt cac aca aga act ggg caa tgt gag			2856
Asn Gly Ser Phe Ser Glu Asp Cys His Thr Arg Thr Gly Gln Cys Glu			
900	905	910	
tgc aga ccc aat gtt cag ggg cgg cac tgt gac gag tgt aag cct gaa			2904
Cys Arg Pro Asn Val Gln Gly Arg His Cys Asp Glu Cys Lys Pro Glu			
915	920	925	
acc ttt ggc ctg caa ctg gga agg ggt tgt ctg ccc tgc aac tgc aat			2952
Thr Phe Gly Leu Gln Leu Gly Arg Gly Cys Leu Pro Cys Asn Cys Asn			
930	935	940	
tct ttt ggg tct aag tcc ttt gac tgt gaa gca agt ggg cag tgc tgg			3000
Ser Phe Gly Ser Lys Ser Phe Asp Cys Glu Ala Ser Gly Gln Cys Trp			
945	950	955	960
tgc cag cct gga gta gca ggg aag aaa tgt gac cgt tgt gcc cat ggc			3048
Cys Gln Pro Gly Val Ala Gly Lys Lys Cys Asp Arg Cys Ala His Gly			
965	970	975	
tac ttc aac ttc caa gaa gga ggc tgc ata gct tgt gac tgt tct cat			3096
Tyr Phe Asn Phe Gln Glu Gly Gly Cys Ile Ala Cys Asp Cys Ser His			
980	985	990	

ctg ggc aac aac tgt gac cca aaa act ggc caa tgc att tgc cca ccc 3144

Leu Gly Asn Asn Cys Asp Pro Lys Thr Gly Gln Cys Ile Cys Pro Pro

995

1000

1005

aat acc act gga gaa aag tgt tct gag tgt ctt ccc aac acc tgg 3189

Asn Thr Thr Gly Glu Lys Cys Ser Glu Cys Leu Pro Asn Thr Trp

1010

1015

1020

ggt cac agc att gtc acc ggc tgt aag gtt tgt aac tgc agc act 3234

Gly His Ser Ile Val Thr Gly Cys Lys Val Cys Asn Cys Ser Thr

1025

1030

1035

gtg ggg tcc ttg gct tct cag tgc aat gta aac acg ggc cag tgc 3279

Val Gly Ser Leu Ala Ser Gln Cys Asn Val Asn Thr Gly Gln Cys

1040

1045

1050

agc tgt cat cca aaa ttc tct ggt atg aaa tgc tca gag tgc agc 3324

Ser Cys His Pro Lys Phe Ser Gly Met Lys Cys Ser Glu Cys Ser

1055

1060

1065

cga ggt cac tgg aac tat cct ctc tgc act cta tgt gac tgc ttc 3369

Arg Gly His Trp Asn Tyr Pro Leu Cys Thr Leu Cys Asp Cys Phe

1070

1075

1080

ctt cca ggc aca gat gcc acg act tgt gat ctg gag act agg aaa 3414

Leu Pro Gly Thr Asp Ala Thr Thr Cys Asp Leu Glu Thr Arg Lys

1085	1090	1095	
tgc tcc tgt agt gat caa act	gga cag tgc agc tgt	aag gtg aat	3459
Cys Ser Cys Ser Asp Gln Thr	Gly Gln Cys Ser Cys	Lys Val Asn	
1100	1105	1110	
gtg gaa ggc gtc cac tgt gac	agg tgc cgg cct ggc	aaa ttt gga	3504
Val Glu Gly Val His Cys Asp	Arg Cys Arg Pro Gly	Lys Phe Gly	
1115	1120	1125	
cta gat gcc aag aac cca ctt	ggc tgc agc agc tgc	tac tgc ttt	3549
Leu Asp Ala Lys Asn Pro Leu	Gly Cys Ser Ser Cys	Tyr Cys Phe	
1130	1135	1140	
gga gtt act agt caa tgc tct	gaa gca aag ggg ctg	atc cgt acg	3594
Gly Val Thr Ser Gln Cys Ser	Glu Ala Lys Gly Leu	Ile Arg Thr	
1145	1150	1155	
tgg gtg act ttg agt gat gaa	cag acc att cta cct	ctg gtg gat	3639
Trp Val Thr Leu Ser Asp Glu	Gln Thr Ile Leu Pro	Leu Val Asp	
1160	1165	1170	
gag gcc ctg cag cac acg act	acc aaa ggc att gct	ttc cag aaa	3684
Glu Ala Leu Gln His Thr Thr	Thr Lys Gly Ile Ala	Phe Gln Lys	
1175	1180	1185	

cca gag att gtt gca aag atg gat gaa gtc agg caa gag ctc cat	3729
Pro Glu Ile Val Ala Lys Met Asp Glu Val Arg Gln Glu Leu His	
1190 1195 1200	
ttg gaa cct ttt tac tgg aaa ctc cca caa caa ttt gaa ggg aaa	3774
Leu Glu Pro Phe Tyr Trp Lys Leu Pro Gln Gln Phe Glu Gly Lys	
1205 1210 1215	
aag ttg atg gct tat ggt ggc aaa ctc aag tat gcc atc tat ttt	3819
Lys Leu Met Ala Tyr Gly Gly Lys Leu Lys Tyr Ala Ile Tyr Phe	
1220 1225 1230	
gag gct cgg gat gag aca ggc ttt gcc aca tat aaa cct caa gtt	3864
Glu Ala Arg Asp Glu Thr Gly Phe Ala Thr Tyr Lys Pro Gln Val	
1235 1240 1245	
atc att cga ggt gga act cct act cat gct aga att att acc aga	3909
Ile Ile Arg Gly Gly Thr Pro Thr His Ala Arg Ile Ile Thr Arg	
1250 1255 1260	
cac atg gct gcc cct ctc att ggc cag ttg aca cgg cat gaa ata	3954
His Met Ala Ala Pro Leu Ile Gly Gln Leu Thr Arg His Glu Ile	
1265 1270 1275	
gaa atg aca gag aaa gaa tgg aaa tat tat ggt gat gat cct cga	3999
Glu Met Thr Glu Lys Glu Trp Lys Tyr Tyr Gly Asp Asp Pro Arg	

1280	1285	1290	
atc agt aga act gtg acc cgt	gaa gac ttc ttg gat	ata cta tat	4044
Ile Ser Arg Thr Val Thr Arg	Glu Asp Phe Leu Asp	Ile Leu Tyr	
1295	1300	1305	
gat att cac tat atc ctt atc	aag gct act tat gga	aac gtt gtg	4089
Asp Ile His Tyr Ile Leu Ile	Lys Ala Thr Tyr Gly	Asn Val Val	
1310	1315	1320	
aga caa ago cgo att tct gaa	atc tcc atg gaa gta	gct gaa cca	4134
Arg Gln Ser Arg Ile Ser Glu	Ile Ser Met Glu Val	Ala Glu Pro	
1325	1330	1335	
gga cat gta tta gca ggg ago	cca cca gca cac ttg	ata gaa aga	4179
Gly His Val Leu Ala Gly Ser	Pro Pro Ala His Leu	Ile Glu Arg	
1340	1345	1350	
tgc gat tgc cct cct ggc tat	tct ggc ttg tct tgt	gag acg tgt	4224
Cys Asp Cys Pro Pro Gly Tyr	Ser Gly Leu Ser Cys	Glu Thr Cys	
1355	1360	1365	
gca cca gga ttt tac cga ctt	cgt tct gaa cca ggt	ggg cgg act	4269
Ala Pro Gly Phe Tyr Arg Leu	Arg Ser Glu Pro Gly	Gly Arg Thr	
1370	1375	1380	

cct gga cca acc tta ggg acc tgt gtt ccc tgc caa tgt aat gga 4314

Pro Gly Pro Thr Leu Gly Thr Cys Val Pro Cys Gln Cys Asn Gly

1385

1390

1395

cac ago agt cag tgt gat cct gag acc tca gta tgc cag aat tgt 4359

His Ser Ser Gln Cys Asp Pro Glu Thr Ser Val Cys Gln Asn Cys

1400

1405

1410

cag cat cac act gct ggt gac ttc tgt gag cgc tgt gcc ott ggc 4404

Gln His His Thr Ala Gly Asp Phe Cys Glu Arg Cys Ala Leu Gly

1415

1420

1425

tac tat gga atc gtc agg gga ttg cca aat gac tgc caa cca tgt 4449

Tyr Tyr Gly Ile Val Arg Gly Leu Pro Asn Asp Cys Gln Pro Cys

1430

1435

1440

gct tgt cct ctg att tog ccc ago aac aat ttc agc ccc tot tgt 4494

Ala Cys Pro Leu Ile Ser Pro Ser Asn Asn Phe Ser Pro Ser Cys

1445

1450

1455

gta ttg gaa ggt ctg gaa gat tac cgt tgc acc gcc tgc cca agg 4539

Val Leu Glu Gly Leu Glu Asp Tyr Arg Cys Thr Ala Cys Pro Arg

1460

1465

1470

ggc tat gaa gga cag tac tgt gaa agg tgt gcc cca ggc tat act 4584

Gly Tyr Glu Gly Gln Tyr Cys Glu Arg Cys Ala Pro Gly Tyr Thr

1475	1480	1485	
ggc agc cca agc agc ccc gga	ggc tcc tgc caa gaa	tgt gag tgt	4629
Gly Ser Pro Ser Ser Pro Gly	Gly Ser Cys Gln Glu	Cys Glu Cys	
1490	1495	1500	
gac cct tat ggc tcc cta ccg	gtt ccc tgt gac cgg	gtc aca gga	4674
Asp Pro Tyr Gly Ser Leu Pro	Val Pro Cys Asp Arg	Val Thr Gly	
1505	1510	1515	
ctc tgc acg tgc cgc cct gga	gcc aca gga agg aag	tgt gat ggc	4719
Leu Cys Thr Cys Arg Pro Gly	Ala Thr Gly Arg Lys	Cys Asp Gly	
1520	1525	1530	
tgc gag cac tgg cat gca cgc	gag ggt gca gag tgt	gtc ttt tgt	4764
Cys Glu His Trp His Ala Arg	Glu Gly Ala Glu Cys	Val Phe Cys	
1535	1540	1545	
gga gac gag tgt aca ggc ctt	ctt ctt ggt gac ctg	gct cgt cta	4809
Gly Asp Glu Cys Thr Gly Leu	Leu Leu Gly Asp Leu	Ala Arg Leu	
1550	1555	1560	
gag cag atg acc atg aac atc	aac ctc acg ggc cca	ctg cct gct	4854
Glu Gln Met Thr Met Asn Ile	Asn Leu Thr Gly Pro	Leu Pro Ala	
1565	1570	1575	

cca tat	aaa att	ctg tat	ggc	ctt gaa	aat aca	act	cag gaa	ctc	4899
Pro Tyr	Lys Ile	Leu Tyr	Gly	Leu Glu	Asn Thr	Thr	Gln Glu	Leu	
1580		1585		1590					
aag cac	ctg cta	tca cgc	caa	cgg gca	cca gag	agg	ctc att	cag	4944
Lys His	Leu Leu	Ser Pro	Gln	Arg Ala	Pro Glu	Arg	Leu Ile	Gln	
1595		1600		1605					
ttg gca	gag ggc	aac gtg	aac	aca ctt	gtg atg	gaa	aca aat	gag	4989
Leu Ala	Glu Gly	Asn Val	Asn	Thr Leu	Val Met	Glu	Thr Asn	Glu	
1610		1615		1620					
ctg cta	acc aga	gca acc	aaa	gtg aca	gca gat	ggc	gag caa	aca	5034
Leu Leu	Thr Arg	Ala Thr	Lys	Val Thr	Ala Asp	Gly	Glu Gln	Thr	
1625		1630		1635					
gga caa	gat gct	gag agg	acc	aac tcc	aga gca	gaa	tcc ttg	gaa	5079
Gly Gln	Asp Ala	Glu Arg	Thr	Asn Ser	Arg Ala	Glu	Ser Leu	Glu	
1640		1645		1650					
gaa ttc	att aaa	ggg ctt	gtc	cag gat	gct gaa	gcc	ata aat	gaa	5124
Glu Phe	Ile Lys	Gly Leu	Val	Gln Asp	Ala Glu	Ala	Ile Asn	Glu	
1655		1660		1665					
aaa gct	gta aaa	cta aat	gaa	acc tta	gga aat	caa	gat aag	aca	5169
Lys Ala	Val Lys	Leu Asn	Glu	Thr Leu	Gly Asn	Gln	Asp Lys	Thr	

1670	1675	1680	
gca gag aga aac ttg gag gag ctt caa aag gaa atc gac cgg atg			5214
Ala Glu Arg Asn Leu Glu Glu Leu Gln Lys Glu Ile Asp Arg Met			
1685	1690	1695	
ctg aag gaa ctg aga agt aaa gat ctt caa aca cag aag gaa gtt			5259
Leu Lys Glu Leu Arg Ser Lys Asp Leu Gln Thr Gln Lys Glu Val			
1700	1705	1710	
gct gag gat gag ctc gtg gca gca gaa ggc ctt ctg aag aga gta			5304
Ala Glu Asp Glu Leu Val Ala Ala Glu Gly Leu Leu Lys Arg Val			
1715	1720	1725	
aac aag ctg ttt gga gag ccc aga gcc cag aat gaa gat atg gaa			5349
Asn Lys Leu Phe Gly Glu Pro Arg Ala Gln Asn Glu Asp Met Glu			
1730	1735	1740	
aag gat ctc cag cag aaa ctg gca gag tac aag aac aaa ctt gat			5394
Lys Asp Leu Gln Gln Lys Leu Ala Glu Tyr Lys Asn Lys Leu Asp			
1745	1750	1755	
gat gct tgg gat cta ttg aga gaa gcc act gat aaa acc cga gat			5439
Asp Ala Trp Asp Leu Leu Arg Glu Ala Thr Asp Lys Thr Arg Asp			
1760	1765	1770	

gct aat cgt ttg tot gct gcc aat caa aaa aac atg acc ata ctg	5484
Ala Asn Arg Leu Ser Ala Ala Asn Gln Lys Asn Met Thr Ile Leu	
1775 1780 1785	
 gag aca aag aag gag got att gaa ggt agc aaa cga caa ata gag	5529
Glu Thr Lys Lys Glu Ala Ile Glu Gly Ser Lys Arg Gln Ile Glu	
1790 1795 1800	
 aac act tta aag gaa ggc aat gac atc ctt gat gaa gcc aat caa	5574
Asn Thr Leu Lys Glu Gly Asn Asp Ile Leu Asp Glu Ala Asn Gln	
1805 1810 1815	
 ctc tta ggt gaa atc aac tca gtc ata gat tat gtc gac gac att	5619
Leu Leu Gly Glu Ile Asn Ser Val Ile Asp Tyr Val Asp Asp Ile	
1820 1825 1830	
 aaa act aag ttg cca cca atg tcc gag gag ctg agt gac aaa ata	5664
Lys Thr Lys Leu Pro Pro Met Ser Glu Glu Leu Ser Asp Lys Ile	
1835 1840 1845	
 gat gac ctc gcc cag gaa ata aag gac aga agg ctt gct gag aag	5709
Asp Asp Leu Ala Gln Glu Ile Lys Asp Arg Arg Leu Ala Glu Lys	
1850 1855 1860	
 gtg ttc cag got gag agc cat gct gct cag ctg aac gac tcg tot	5754
Val Phe Gln Ala Glu Ser His Ala Ala Gln Leu Asn Asp Ser Ser	

1865	1870	1875	
gct gta ctt gat gga atc ctg gat gag gct aag aac atc tct ttc			5799
Ala Val Leu Asp Gly Ile Leu Asp Glu Ala Lys Asn Ile Ser Phe			
1880	1885	1890	
aat gcc acg gca gcc ttc aga gct tac agt aat att aaa gac tac			5844
Asn Ala Thr Ala Ala Phe Arg Ala Tyr Ser Asn Ile Lys Asp Tyr			
1895	1900	1905	
att gat gaa gct gag aaa gtg gcc aga gaa gcc aaa gag ctt gcc			5889
Ile Asp Glu Ala Glu Lys Val Ala Arg Glu Ala Lys Glu Leu Ala			
1910	1915	1920	
caa ggg gct aca aaa ctg gca aca agt oct cag ggc tta tta aaa			5934
Gln Gly Ala Thr Lys Leu Ala Thr Ser Pro Gln Gly Leu Leu Lys			
1925	1930	1935	
gaa gat gcc aaa ggc tcc ctt cag aaa agc ttc agg atc ctc aat			5979
Glu Asp Ala Lys Gly Ser Leu Gln Lys Ser Phe Arg Ile Leu Asn			
1940	1945	1950	
gaa gcc aag aag cta gca aac gat gtg aaa gga aat cac aat gat			6024
Glu Ala Lys Lys Leu Ala Asn Asp Val Lys Gly Asn His Asn Asp			
1955	1960	1965	

cta aat	gac ctg aaa acc agg	tta gaa act gct gac	ctt aga aac	6069
Leu Asn	Asp Leu Lys Thr Arg	Leu Glu Thr Ala Asp	Leu Arg Asn	
1970	1975	1980		
agt gga	ctt cta gga got cta	aat gac acc atg gac	aag tta tca	6114
Ser Gly	Leu Leu Gly Ala Leu	Asn Asp Thr Met Asp	Lys Leu Ser	
1985	1990	1995		
gcc att	aca aat gac acg gct	gct aaa ctg cag gct	gtc aaa gag	6159
Ala Ile	Thr Asn Asp Thr Ala	Ala Lys Leu Gln Ala	Val Lys Glu	
2000	2005	2010		
aaa gcc	aga gaa gcc aat gac	aca gca aaa got gtc	ctg gcc cag	6204
Lys Ala	Arg Glu Ala Asn Asp	Thr Ala Lys Ala Val	Leu Ala Gln	
2015	2020	2025		
gtt aag	gac ctg cat cag aac	cta gat ggc ctg aag	caa aac tac	6249
Val Lys	Asp Leu His Gln Asn	Leu Asp Gly Leu Lys	Gln Asn Tyr	
2030	2035	2040		
aat aaa	ctg gca gac agc gtg	gcc aaa acg aac gct	gtg gtg aaa	6294
Asn Lys	Leu Ala Asp Ser Val	Ala Lys Thr Asn Ala	Val Val Lys	
2045	2050	2055		
gat cct	tcc aaa aac aaa atc	att gca gat gca ggc	act tcc gtg	6339
Asp Pro	Ser Lys Asn Lys Ile	Ile Ala Asp Ala Gly	Thr Ser Val	

2060	2065	2070	
aga aat cta gaa cag gaa gct	gac cgg cta atc gac	aaa ctc aag	6384
Arg Asn Leu Glu Gln Glu Ala	Asp Arg Leu Ile Asp	Lys Leu Lys	
2075	2080	2085	
ccc atc aag gag ctt gag gac	aac cta aag aaa aac	att tct gaa	6429
Pro Ile Lys Glu Leu Glu Asp	Asn Leu Lys Lys Asn	Ile Ser Glu	
2090	2095	2100	
ata aag gaa ctg atc aac caa	gct cgg aaa caa gct	aac tct atc	6474
Ile Lys Glu Leu Ile Asn Gln	Ala Arg Lys Gln Ala	Asn Ser Ile	
2105	2110	2115	
aaa gta tct gtt tct tcg gga	ggc gac tgt gtt cgg	aca tac agg	6519
Lys Val Ser Val Ser Ser Gly	Gly Asp Cys Val Arg	Thr Tyr Arg	
2120	2125	2130	
cca gaa atc aag aaa gga agc	tac aat aac atc gtt	gtc cat gtc	6564
Pro Glu Ile Lys Lys Gly Ser	Tyr Asn Asn Ile Val	Val His Val	
2135	2140	2145	
aag acc gct gtt gcc gac aac	ctc ctt ttt tat ctt	gga agt gcc	6609
Lys Thr Ala Val Ala Asp Asn	Leu Leu Phe Tyr Leu	Gly Ser Ala	
2150	2155	2160	

aaa ttt att gac ttt ctt gct ata gaa atg cgc aaa ggc aaa gtc 6654

Lys Phe Ile Asp Phe Leu Ala Ile Glu Met Arg Lys Gly Lys Val

2165

2170

2175

agc ttc oto tgg att gtt ggc tct gga gtt ggc cga gta ggg ttt 6699

Ser Phe Leu Trp Ile Val Gly Ser Gly Val Gly Arg Val Gly Phe

2180

2185

2190

cca gac ttg acc atc gac gac tcc tat tgg tac cgt att gaa gca 6744

Pro Asp Leu Thr Ile Asp Asp Ser Tyr Trp Tyr Arg Ile Glu Ala

2195

2200

2205

tca aga acg gga aga aat gga tct att tct gtg aga gct tta gat 6789

Ser Arg Thr Gly Arg Asn Gly Ser Ile Ser Val Arg Ala Leu Asp

2210

2215

2220

gga ccc aaa gcc agt atg gta ccc agc acc tac cat tca gtg tct 6834

Gly Pro Lys Ala Ser Met Val Pro Ser Thr Tyr His Ser Val Ser

2225

2230

2235

cct ccc ggg tat act atc cta gat gtg gat gca aat gca atg ctg 6879

Pro Pro Gly Tyr Thr Ile Leu Asp Val Asp Ala Asn Ala Met Leu

2240

2245

2250

ttt gtt ggt ggc ctg acc gga aaa ata aag aag gcc gat gct gta 6924

Phe Val Gly Gly Leu Thr Gly Lys Ile Lys Lys Ala Asp Ala Val

2255	2260	2265	
ogt gtg atc acc ttc acc ggc	tgt atg gga gaa aca	tac ttt gac	6969
Arg Val Ile Thr Phe Thr Gly	Cys Met Gly Glu Thr	Tyr Phe Asp	
2270	2275	2280	
aac aaa cct ata ggt tta tgg	aac ttc cgg gag aaa	gaa ggc gac	7014
Asn Lys Pro Ile Gly Leu Trp	Asn Phe Arg Glu Lys	Glu Gly Asp	
2285	2290	2295	
tgt aag gga tgt act gtc agc	cca caa gtg gaa gat	agt gag ggg	7059
Cys Lys Gly Cys Thr Val Ser	Pro Gln Val Glu Asp	Ser Glu Gly	
2300	2305	2310	
act att cag ttt gat ggt gaa	ggc tat gca tta gtg	agc cgg ccc	7104
Thr Ile Gln Phe Asp Gly Glu	Gly Tyr Ala Leu Val	Ser Arg Pro	
2315	2320	2325	
atc cgc tgg tac ccc aac atc	tcc aca gtc atg ttc	aag ttc cgg	7149
Ile Arg Trp Tyr Pro Asn Ile	Ser Thr Val Met Phe	Lys Phe Arg	
2330	2335	2340	
aca ttt toa tca agt gct ctc	ctg atg tat ctt gcc	aca cga gac	7194
Thr Phe Ser Ser Ser Ala Leu	Leu Met Tyr Leu Ala	Thr Arg Asp	
2345	2350	2355	

ctg aaa gat ttc atg agt gta gag ctc agt gat gga cat gtg aaa 7239

Leu Lys Asp Phe Met Ser Val Glu Leu Ser Asp Gly His Val Lys

2360

2365

2370

gtc agc tat gac ctg ggc tca gga atg act tcc gtt gtc agc aat 7284

Val Ser Tyr Asp Leu Gly Ser Gly Met Thr Ser Val Val Ser Asn

2375

2380

2385

caa aac cat aat gat ggg aaa tgg aaa gca ttc acg ctg tcg cgg 7329

Gln Asn His Asn Asp Gly Lys Trp Lys Ala Phe Thr Leu Ser Arg

2390

2395

2400

att cag aaa caa gcc aac ata tcg att gtc gac atc gat tct aac 7374

Ile Gln Lys Gln Ala Asn Ile Ser Ile Val Asp Ile Asp Ser Asn

2405

2410

2415

cag gag gag aat gta gct act tca tct tct gga aac aac ttt ggt 7419

Gln Glu Gly Asn Val Ala Thr Ser Ser Ser Gly Asn Asn Phe Gly

2420

2425

2430

ctt gac ttg aaa gca gat gac aaa ata tat ttt ggt ggc ctg cca 7464

Leu Asp Leu Lys Ala Asp Asp Lys Ile Tyr Phe Gly Gly Leu Pro

2435

2440

2445

act ctg aga aac ttg agt atg aaa gca agg cca gaa gtc aat gtg 7509

Thr Leu Arg Asn Leu Ser Met Lys Ala Arg Pro Glu Val Asn Val

2450	2455	2460	
aag aaa tac tcc ggc tgc ctc	aaa gat att gaa att	tca aga aca	7554
Lys Lys Tyr Ser Gly Cys Leu	Lys Asp Ile Glu Ile	Ser Arg Thr	
2465	2470	2475	
cct tac aat ata ctc agc agc	cct gat tat gtt ggt	gtg acc aaa	7599
Pro Tyr Asn Ile Leu Ser Ser	Pro Asp Tyr Val Gly	Val Thr Lys	
2480	2485	2490	
ggc tgt tca ctg gag aat gtt	aat aca gtt agt ttc	ccc aag cct	7644
Gly Cys Ser Leu Glu Asn Val	Asn Thr Val Ser Phe	Pro Lys Pro	
2495	2500	2505	
ggt ttt gtg gag ctt gcc gct	gtg tct att gat gtt	gga aca gaa	7689
Gly Phe Val Glu Leu Ala Ala	Val Ser Ile Asp Val	Gly Thr Glu	
2510	2515	2520	
atc aat ctg tcc ttt agt acc	agg aac gag tct ggg	atc att ctc	7734
Ile Asn Leu Ser Phe Ser Thr	Arg Asn Glu Ser Gly	Ile Ile Leu	
2525	2530	2535	
ttg gga agt gga ggg aca ctc	aca cca ccc agg aga	aaa cgg aga	7779
Leu Gly Ser Gly Gly Thr Leu	Thr Pro Pro Arg Arg	Lys Arg Arg	
2540	2545	2550	

caa acc aca cag gct tat tat gcc ata ttc ctc aac aag ggc cgc 7824

Gln Thr Thr Gln Ala Tyr Tyr Ala Ile Phe Leu Asn Lys Gly Arg

2555

2560

2565

ttg gaa gtg cat ctc tcc tcg ggg aca cgg aca atg agg aaa att 7869

Leu Glu Val His Leu Ser Ser Gly Thr Arg Thr Met Arg Lys Ile

2570

2575

2580

gtc atc aaa ccg gag cca aat ttg ttt cat gat ggg aga gaa cat 7914

Val Ile Lys Pro Glu Pro Asn Leu Phe His Asp Gly Arg Glu His

2585

2590

2595

tct gtc cac gta gaa aga acc aga ggc atc ttc act gtt caa att 7959

Ser Val His Val Glu Arg Thr Arg Gly Ile Phe Thr Val Gln Ile

2600

2605

2610

gat gaa gac aga aga cat atc caa aac ctg aca gag gaa cag ccc 8004

Asp Glu Asp Arg Arg His Ile Gln Asn Leu Thr Glu Glu Gln Pro

2615

2620

2625

atc gaa gtg aaa aag ctc ttt gtc ggg ggt gct cct cct gaa ttt 8049

Ile Glu Val Lys Lys Leu Phe Val Gly Gly Ala Pro Pro Glu Phe

2630

2635

2640

cag ccc tcc cca ctc agg aat att ccg gcc ttt caa ggc tgt gtg 8094

Gln Pro Ser Pro Leu Arg Asn Ile Pro Ala Phe Gln Gly Cys Val

2645	2650	2655	
tgg aac ctt gtt att aac tcc	atc ccc atg gac ttt	gog cag cct	8139
Trp Asn Leu Val Ile Asn Ser	Ile Pro Met Asp Phe	Ala Gln Pro	
2660	2665	2670	
ata gcc ttc aaa aat gcc gac	att ggt cgc tgt acc	tat caa aag	8184
Ile Ala Phe Lys Asn Ala Asp	Ile Gly Arg Cys Thr	Tyr Gln Lys	
2675	2680	2685	
ccc cgg gaa gat gag agt gaa	gca gtt cca gct gaa	gtt att gtc	8229
Pro Arg Glu Asp Glu Ser Glu	Ala Val Pro Ala Glu	Val Ile Val	
2690	2695	2700	
cag cct cag tgc gtg ccc acc	cct gcc ttc cct ttc	cca gtc ccc	8274
Gln Pro Gln Ser Val Pro Thr	Pro Ala Phe Pro Phe	Pro Val Pro	
2705	2710	2715	
acc atg gtg cat ggc cct tgt	gtt gca gaa tca gaa	cca gct ctt	8319
Thr Met Val His Gly Pro Cys	Val Ala Glu Ser Glu	Pro Ala Leu	
2720	2725	2730	
ctg aca ggg agc aag cag ttt	ggg ctt tcc aga aac	agc cac att	8364
Leu Thr Gly Ser Lys Gln Phe	Gly Leu Ser Arg Asn	Ser His Ile	
2735	2740	2745	

gca att gtc ttt gat gac acc	aaa gtt aaa aac cgc	cto acc att	8409
Ala Ile Val Phe Asp Asp Thr	Lys Val Lys Asn Arg	Leu Thr Ile	
2750	2755	2760	
gag ctg gag gta cga act gaa	gct gaa tca ggc ttg	cto ttc tac	8454
Glu Leu Glu Val Arg Thr Glu	Ala Glu Ser Gly Leu	Leu Phe Tyr	
2765	2770	2775	
atg ggt cgg atc aat cat gct	gat ttt ggt act gtt	cag ctg agg	8499
Met Gly Arg Ile Asn His Ala	Asp Phe Gly Thr Val	Gln Leu Arg	
2780	2785	2790	
aat ggg ttc ccg ttc ttc agt	tat gat ttg ggg agt	ggg agc acc	8544
Asn Gly Phe Pro Phe Phe Ser	Tyr Asp Leu Gly Ser	Gly Ser Thr	
2795	2800	2805	
aga acc atg atc ccc aca aaa	atc aac gat ggt cag	tgg cac aag	8589
Arg Thr Met Ile Pro Thr Lys	Ile Asn Asp Gly Gln	Trp His Lys	
2810	2815	2820	
att aag att gtg aga gtg aag	cag gag gga att ctt	tat gtg gat	8634
Ile Lys Ile Val Arg Val Lys	Gln Glu Gly Ile Leu	Tyr Val Asp	
2825	2830	2835	
gat gcc tcc agc caa acc atc	agt ccc aag aaa gcc	gac atc ctg	8679
Asp Ala Ser Ser Gln Thr Ile	Ser Pro Lys Lys Ala	Asp Ile Leu	

2840	2845	2850	
gat gtc ggg ggg att ctg tat gtc ggt gga ttg ccg atc aac tat			8724
Asp Val Gly Gly Ile Leu Tyr Val Gly Gly Leu Pro Ile Asn Tyr			
2855	2860	2865	
acc aca cgc aga att ggt cca gtg act tac agc ctg gat ggc tgt			8769
Thr Thr Arg Arg Ile Gly Pro Val Thr Tyr Ser Leu Asp Gly Cys			
2870	2875	2880	
gtt agg aat ctt cac atg gaa caa gcc cct gtt gat ctg gac cag			8814
Val Arg Asn Leu His Met Glu Gln Ala Pro Val Asp Leu Asp Gln			
2885	2890	2895	
cot acc tcc agc ttt cac gtt ggg aca tgc ttt gcg aat gca gag			8859
Pro Thr Ser Ser Phe His Val Gly Thr Cys Phe Ala Asn Ala Glu			
2900	2905	2910	
agt ggg act tac ttt gat gga acc ggt ttt ggt aaa gca gtt ggt			8904
Ser Gly Thr Tyr Phe Asp Gly Thr Gly Phe Gly Lys Ala Val Gly			
2915	2920	2925	
ggg ttc atc gtt gga ttg gac ott ctt gtg gaa ttt gaa ttc cgt			8949
Gly Phe Ile Val Gly Leu Asp Leu Leu Val Glu Phe Glu Phe Arg			
2930	2935	2940	

acc aca aga ccc act ggg gtc ctc ctg ggg atc agc agt cag aag 8994

Thr Thr Arg Pro Thr Gly Val Leu Leu Gly Ile Ser Ser Gln Lys

2945

2950

2955

atg gat gga atg ggt att gaa atg atc gac gag aag ctt atg ttc 9039

Met Asp Gly Met Gly Ile Glu Met Ile Asp Glu Lys Leu Met Phe

2960

2965

2970

cac gtg gat aat ggc gct ggc cga tto act gca att tat gat gct 9084

His Val Asp Asn Gly Ala Gly Arg Phe Thr Ala Ile Tyr Asp Ala

2975

2980

2985

gag atc cca ggc cac atg tgc aat gga cag tgg tat aaa gtc act 9129

Glu Ile Pro Gly His Met Cys Asn Gly Gln Trp Tyr Lys Val Thr

2990

2995

3000

gcc aag aag atc aaa aac cgt ctt gag ctg gtg gta gat ggg aac 9174

Ala Lys Lys Ile Lys Asn Arg Leu Glu Leu Val Val Asp Gly Asn

3005

3010

3015

cag gtg gat gcc cag agc cca aac tca gca tcg aca tca gct gat 9219

Gln Val Asp Ala Gln Ser Pro Asn Ser Ala Ser Thr Ser Ala Asp

3020

3025

3030

aca aac gac cct gtt ttc gtt ggc ggt ttc cca ggt ggc ctc aat 9264

Thr Asn Asp Pro Val Phe Val Gly Gly Phe Pro Gly Gly Leu Asn

3035 3040 3045
cag ttt ggc ctg acc acc aac att agg ttc cga ggc tgc atc cga 9309
Gln Phe Gly Leu Thr Thr Asn Ile Arg Phe Arg Gly Cys Ile Arg
3050 3055 3060
tct ctg aag ctc acc aaa ggc act gca aac cgc tgg agg tta att 9354
Ser Leu Lys Leu Thr Lys Gly Thr Ala Asn Arg Trp Arg Leu Ile
3065 3070 3075
ttg cca agg ccc tgg aac tgaggggtgt tcaacctgta tcatgccga 9402
Leu Pro Arg Pro Trp Asn
3080
ctaccttaata aagatagttc aatcctgagg agaattcatc aaaacaagta tatcaagtta 9462
aacaatatac actcctatca tattaataaa actaatgtgc agcggccgc 9511

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<211> 3084

<212> PRT

<213> Mus musculus

<400> 6

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Val Leu Asn Leu Ala Ser Asn Ala Leu Ile Thr Thr Asn Ala Thr Cys

20 25 30

Gly Glu Lys Gly Pro Glu Met Tyr Cys Lys Leu Val Glu His Val Pro

35 40 45

Gly Gln Pro Val Arg Asn Pro Gln Cys Arg Ile Cys Asn Gln Asn Ser

50 55 60

Ser Asn Pro Tyr Gln Arg His Pro Ile Thr Asn Ala Ile Asp Gly Lys

65 70 75 80

Asn Thr Trp Trp Gln Ser Pro Ser Ile Lys Asn Gly Val Glu Tyr His

85 90 95

Tyr Val Thr Ile Thr Leu Asp Leu Gln Gln Val Phe Gln Ile Ala Tyr

100 105 110

Val Ile Val Lys Ala Ala Asn Ser Pro Arg Pro Gly Asn Trp Ile Leu
115 120 125

Glu Arg Ser Leu Asp Asp Val Glu Tyr Lys Pro Trp Gln Tyr His Ala
130 135 140

Val Thr Asp Thr Glu Cys Leu Thr Leu Tyr Asn Ile Tyr Pro Arg Thr
145 150 155 160

Gly Pro Pro Ser Tyr Ala Lys Asp Asp Glu Val Ile Cys Thr Ser Phe
165 170 175

Tyr Ser Lys Ile His Pro Leu Glu Asn Gly Glu Ile His Ile Ser Leu
180 185 190

Ile Asn Gly Arg Pro Ser Ala Asp Asp Pro Ser Pro Glu Leu Leu Glu
195 200 205

Phe Thr Ser Ala Arg Tyr Ile Arg Leu Arg Phe Gln Arg Ile Arg Thr

210

215

220

Leu Asn Ala Asp Leu Met Met Phe Ala His Lys Asp Pro Arg Glu Ile

225

230

235

240

Asp Pro Ile Val Thr Arg Arg Tyr Tyr Tyr Ser Val Lys Asp Ile Ser

245

250

255

Val Gly Gly Met Cys Ile Cys Tyr Gly His Ala Arg Ala Cys Pro Leu

260

265

270

Asp Pro Ala Thr Asn Lys Ser Arg Cys Glu Cys Glu His Asn Thr Cys

275

280

285

Gly Glu Ser Cys Asp Arg Cys Cys Pro Gly Phe His Gln Lys Pro Trp

290

295

300

Arg Ala Gly Thr Phe Leu Thr Lys Ser Glu Cys Glu Ala Cys Asn Cys

305

310

315

320

His Gly Lys Ala Glu Glu Cys Tyr Tyr Asp Glu Thr Val Ala Ser Arg

325

330

335

Asn Leu Ser Leu Asn Ile His Gly Lys Tyr Ile Gly Gly Gly Val Cys

340

345

350

Ile Asn Cys Thr His Asn Thr Ala Gly Ile Asn Cys Glu Thr Cys Val

355

360

365

Asp Gly Phe Phe Arg Pro Lys Gly Val Ser Pro Asn Tyr Pro Arg Pro

370

375

380

Cys Gln Pro Cys His Cys Asp Pro Thr Gly Ser Leu Ser Glu Val Cys

385

390

395

400

Val Lys Asp Glu Lys Tyr Ala Gln Arg Gly Leu Lys Pro Gly Ser Cys

405

410

415

His Cys Lys Thr Gly Phe Gly Gly Val Asn Cys Asp Arg Cys Val Arg

420

425

430

Gly Tyr His Gly Tyr Pro Asp Cys Gln Pro Cys Asn Cys Ser Gly Leu

435

440

445

Gly Ser Thr Asn Glu Asp Pro Cys Val Gly Pro Cys Ser Cys Lys Glu

450

455

460

Asn Val Glu Gly Glu Asp Cys Ser Arg Cys Lys Ser Gly Phe Phe Asn

465

470

475

480

Leu Gln Glu Asp Asn Gln Lys Gly Cys Glu Glu Cys Phe Cys Ser Gly

485

490

495

Val Ser Asn Arg Cys Gln Ser Ser Tyr Trp Thr Tyr Gly Asn Ile Gln

500

505

510

Asp Met Arg Gly Trp Tyr Leu Thr Asp Leu Ser Gly Arg Ile Arg Met

515

520

525

Ala Pro Gln Leu Asp Asn Pro Asp Ser Pro Gln Gln Ile Ser Ile Ser
530 535 540

Asn Ser Glu Ala Arg Lys Ser Leu Leu Asp Gly Tyr Tyr Trp Ser Ala
545 550 555 560

Pro Pro Pro Tyr Leu Gly Asn Arg Leu Pro Ala Val Gly Gly Gln Leu
565 570 575

Ser Phe Thr Ile Ser Tyr Asp Leu Glu Glu Glu Glu Asp Asp Thr Glu
580 585 590

Lys Leu Leu Gln Leu Met Ile Ile Phe Glu Gly Asn Asp Leu Arg Ile
595 600 605

Ser Thr Ala Tyr Lys Glu Val Tyr Leu Glu Pro Ser Glu Glu His Val
610 615 620

Glu Glu Val Ser Leu Lys Glu Glu Ala Phe Thr Ile His Gly Thr Asn

625 630 635 640

Leu Pro Val Thr Arg Lys Asp Phe Met Ile Val Leu Thr Asn Leu Gly

645 650 655

Glu Ile Leu Ile Gln Ile Thr Tyr Asn Leu Gly Met Asp Ala Ile Phe

660 665 670

Arg Leu Ser Ser Val Asn Leu Glu Ser Pro Val Pro Tyr Pro Thr Asp

675 680 685

Arg Arg Ile Ala Thr Asp Val Glu Val Cys Gln Cys Pro Pro Gly Tyr

690 695 700

Ser Gly Ser Ser Cys Glu Thr Cys Trp Pro Arg His Arg Arg Val Asn

705 710 715 720

Gly Thr Ile Phe Gly Gly Ile Cys Glu Pro Cys Gln Cys Phe Ala His

725 730 735

Ala Glu Ala Cys Asp Asp Ile Thr Gly Glu Cys Leu Asn Cys Lys Asp
740 745 750

His Thr Gly Gly Pro Tyr Cys Asn Glu Cys Leu Pro Gly Phe Tyr Gly
755 760 765

Asp Pro Thr Arg Gly Ser Pro Glu Asp Cys Gln Pro Cys Ala Cys Pro
770 775 780

Leu Asn Ile Pro Ser Asn Asn Phe Ser Pro Thr Cys His Leu Asp Arg
785 790 795 800

Ser Leu Gly Leu Ile Cys Asp Glu Cys Pro Ile Gly Tyr Thr Gly Pro
805 810 815

Arg Cys Glu Arg Cys Ala Glu Gly Tyr Phe Gly Gln Pro Ser Val Pro
820 825 830

Gly Gly Ser Cys Gln Pro Cys Gln Cys Asn Asp Asn Leu Asp Tyr Ser

835

840

845

Ile Pro Gly Ser Cys Asp Ser Leu Ser Gly Ser Cys Leu Ile Cys Lys

850

855

860

Pro Gly Thr Thr Gly Arg Tyr Cys Glu Leu Cys Ala Asp Gly Tyr Phe

865

870

875

880

Gly Asp Ala Val Asn Thr Lys Asn Cys Gln Pro Cys Arg Cys Asp Ile

885

890

895

Asn Gly Ser Phe Ser Glu Asp Cys His Thr Arg Thr Gly Gln Cys Glu

900

905

910

Cys Arg Pro Asn Val Gln Gly Arg His Cys Asp Glu Cys Lys Pro Glu

915

920

925

Thr Phe Gly Leu Gln Leu Gly Arg Gly Cys Leu Pro Cys Asn Cys Asn

930

935

940

Ser Phe Gly Ser Lys Ser Phe Asp Cys Glu Ala Ser Gly Gln Cys Trp
945 950 955 960

Cys Gln Pro Gly Val Ala Gly Lys Lys Cys Asp Arg Cys Ala His Gly
965 970 975

Tyr Phe Asn Phe Gln Glu Gly Gly Cys Ile Ala Cys Asp Cys Ser His
980 985 990

Leu Gly Asn Asn Cys Asp Pro Lys Thr Gly Gln Cys Ile Cys Pro Pro
995 1000 1005

Asn Thr Thr Gly Glu Lys Cys Ser Glu Cys Leu Pro Asn Thr Trp
1010 1015 1020

Gly His Ser Ile Val Thr Gly Cys Lys Val Cys Asn Cys Ser Thr
1025 1030 1035

Val Gly Ser Leu Ala Ser Gln Cys Asn Val Asn Thr Gly Gln Cys
1040 1045 1050

Ser Cys His Pro Lys Phe Ser Gly Met Lys Cys Ser Glu Cys Ser
1055 1060 1065

Arg Gly His Trp Asn Tyr Pro Leu Cys Thr Leu Cys Asp Cys Phe
1070 1075 1080

Leu Pro Gly Thr Asp Ala Thr Thr Cys Asp Leu Glu Thr Arg Lys
1085 1090 1095

Cys Ser Cys Ser Asp Gln Thr Gly Gln Cys Ser Cys Lys Val Asn
1100 1105 1110

Val Glu Gly Val His Cys Asp Arg Cys Arg Pro Gly Lys Phe Gly
1115 1120 1125

Leu Asp Ala Lys Asn Pro Leu Gly Cys Ser Ser Cys Tyr Cys Phe

1130

1135

1140

Gly Val Thr Ser Gln Cys Ser Glu Ala Lys Gly Leu Ile Arg Thr

1145

1150

1155

Trp Val Thr Leu Ser Asp Glu Gln Thr Ile Leu Pro Leu Val Asp

1160

1165

1170

Glu Ala Leu Gln His Thr Thr Thr Lys Gly Ile Ala Phe Gln Lys

1175

1180

1185

Pro Glu Ile Val Ala Lys Met Asp Glu Val Arg Gln Glu Leu His

1190

1195

1200

Leu Glu Pro Phe Tyr Trp Lys Leu Pro Gln Gln Phe Glu Gly Lys

1205

1210

1215

Lys Leu Met Ala Tyr Gly Gly Lys Leu Lys Tyr Ala Ile Tyr Phe

1220

1225

1230

Glu Ala Arg Asp Glu Thr Gly Phe Ala Thr Tyr Lys Pro Gln Val

1235

1240

1245

Ile Ile Arg Gly Gly Thr Pro Thr His Ala Arg Ile Ile Thr Arg

1250

1255

1260

His Met Ala Ala Pro Leu Ile Gly Gln Leu Thr Arg His Glu Ile

1265

1270

1275

Glu Met Thr Glu Lys Glu Trp Lys Tyr Tyr Gly Asp Asp Pro Arg

1280

1285

1290

Ile Ser Arg Thr Val Thr Arg Glu Asp Phe Leu Asp Ile Leu Tyr

1295

1300

1305

Asp Ile His Tyr Ile Leu Ile Lys Ala Thr Tyr Gly Asn Val Val

1310

1315

1320

Arg Gln Ser Arg Ile Ser Glu Ile Ser Met Glu Val Ala Glu Pro

1325

1330

1335

Gly His Val Leu Ala Gly Ser Pro Pro Ala His Leu Ile Glu Arg

1340

1345

1350

Cys Asp Cys Pro Pro Gly Tyr Ser Gly Leu Ser Cys Glu Thr Cys

1355

1360

1365

Ala Pro Gly Phe Tyr Arg Leu Arg Ser Glu Pro Gly Gly Arg Thr

1370

1375

1380

Pro Gly Pro Thr Leu Gly Thr Cys Val Pro Cys Gln Cys Asn Gly

1385

1390

1395

His Ser Ser Gln Cys Asp Pro Glu Thr Ser Val Cys Gln Asn Cys

1400

1405

1410

Gln His His Thr Ala Gly Asp Phe Cys Glu Arg Cys Ala Leu Gly

1415

1420

1425

Tyr Tyr Gly Ile Val Arg Gly Leu Pro Asn Asp Cys Gln Pro Cys
1430 1435 1440

Ala Cys Pro Leu Ile Ser Pro Ser Asn Asn Phe Ser Pro Ser Cys
1445 1450 1455

Val Leu Glu Gly Leu Glu Asp Tyr Arg Cys Thr Ala Cys Pro Arg
1460 1465 1470

Gly Tyr Glu Gly Gln Tyr Cys Glu Arg Cys Ala Pro Gly Tyr Thr
1475 1480 1485

Gly Ser Pro Ser Ser Pro Gly Gly Ser Cys Gln Glu Cys Glu Cys
1490 1495 1500

Asp Pro Tyr Gly Ser Leu Pro Val Pro Cys Asp Arg Val Thr Gly
1505 1510 1515

Leu Cys Thr Cys Arg Pro Gly Ala Thr Gly Arg Lys Cys Asp Gly

1520

1525

1530

Cys Glu His Trp His Ala Arg Glu Gly Ala Glu Cys Val Phe Cys

1535

1540

1545

Gly Asp Glu Cys Thr Gly Leu Leu Leu Gly Asp Leu Ala Arg Leu

1550

1555

1560

Glu Gln Met Thr Met Asn Ile Asn Leu Thr Gly Pro Leu Pro Ala

1565

1570

1575

Pro Tyr Lys Ile Leu Tyr Gly Leu Glu Asn Thr Thr Gln Glu Leu

1580

1585

1590

Lys His Leu Leu Ser Pro Gln Arg Ala Pro Glu Arg Leu Ile Gln

1595

1600

1605

Leu Ala Glu Gly Asn Val Asn Thr Leu Val Met Glu Thr Asn Glu

1610

1615

1620

Leu Leu Thr Arg Ala Thr Lys Val Thr Ala Asp Gly Glu Gln Thr
1625 1630 1635

Gly Gln Asp Ala Glu Arg Thr Asn Ser Arg Ala Glu Ser Leu Glu
1640 1645 1650

Glu Phe Ile Lys Gly Leu Val Gln Asp Ala Glu Ala Ile Asn Glu
1655 1660 1665

Lys Ala Val Lys Leu Asn Glu Thr Leu Gly Asn Gln Asp Lys Thr
1670 1675 1680

Ala Glu Arg Asn Leu Glu Glu Leu Gln Lys Glu Ile Asp Arg Met
1685 1690 1695

Leu Lys Glu Leu Arg Ser Lys Asp Leu Gln Thr Gln Lys Glu Val
1700 1705 1710

Ala Glu Asp Glu Leu Val Ala Ala Glu Gly Leu Leu Lys Arg Val

1715

1720

1725

Asn Lys Leu Phe Gly Glu Pro Arg Ala Gln Asn Glu Asp Met Glu

1730

1735

1740

Lys Asp Leu Gln Gln Lys Leu Ala Glu Tyr Lys Asn Lys Leu Asp

1745

1750

1755

Asp Ala Trp Asp Leu Leu Arg Glu Ala Thr Asp Lys Thr Arg Asp

1760

1765

1770

Ala Asn Arg Leu Ser Ala Ala Asn Gln Lys Asn Met Thr Ile Leu

1775

1780

1785

Glu Thr Lys Lys Glu Ala Ile Glu Gly Ser Lys Arg Gln Ile Glu

1790

1795

1800

Asn Thr Leu Lys Glu Gly Asn Asp Ile Leu Asp Glu Ala Asn Gln

1805

1810

1815

Leu Leu Gly Glu Ile Asn Ser Val Ile Asp Tyr Val Asp Asp Ile
1820 1825 1830

Lys Thr Lys Leu Pro Pro Met Ser Glu Glu Leu Ser Asp Lys Ile
1835 1840 1845

Asp Asp Leu Ala Gln Glu Ile Lys Asp Arg Arg Leu Ala Glu Lys
1850 1855 1860

Val Phe Gln Ala Glu Ser His Ala Ala Gln Leu Asn Asp Ser Ser
1865 1870 1875

Ala Val Leu Asp Gly Ile Leu Asp Glu Ala Lys Asn Ile Ser Phe
1880 1885 1890

Asn Ala Thr Ala Ala Phe Arg Ala Tyr Ser Asn Ile Lys Asp Tyr
1895 1900 1905

Ile Asp Glu Ala Glu Lys Val Ala Arg Glu Ala Lys Glu Leu Ala

1910	1915	1920
Gln Gly Ala Thr Lys Leu Ala Thr Ser Pro Gln Gly Leu Leu Lys		
1925	1930	1935
Glu Asp Ala Lys Gly Ser Leu Gln Lys Ser Phe Arg Ile Leu Asn		
1940	1945	1950
Glu Ala Lys Lys Leu Ala Asn Asp Val Lys Gly Asn His Asn Asp		
1955	1960	1965
Leu Asn Asp Leu Lys Thr Arg Leu Glu Thr Ala Asp Leu Arg Asn		
1970	1975	1980
Ser Gly Leu Leu Gly Ala Leu Asn Asp Thr Met Asp Lys Leu Ser		
1985	1990	1995
Ala Ile Thr Asn Asp Thr Ala Ala Lys Leu Gln Ala Val Lys Glu		
2000	2005	2010

Lys Ala Arg Glu Ala Asn Asp Thr Ala Lys Ala Val Leu Ala Gln
2015 2020 2025

Val Lys Asp Leu His Gln Asn Leu Asp Gly Leu Lys Gln Asn Tyr
2030 2035 2040

Asn Lys Leu Ala Asp Ser Val Ala Lys Thr Asn Ala Val Val Lys
2045 2050 2055

Asp Pro Ser Lys Asn Lys Ile Ile Ala Asp Ala Gly Thr Ser Val
2060 2065 2070

Arg Asn Leu Glu Gln Glu Ala Asp Arg Leu Ile Asp Lys Leu Lys
2075 2080 2085

Pro Ile Lys Glu Leu Glu Asp Asn Leu Lys Lys Asn Ile Ser Glu
2090 2095 2100

Ile Lys Glu Leu Ile Asn Gln Ala Arg Lys Gln Ala Asn Ser Ile

2105

2110

2115

Lys Val Ser Val Ser Ser Gly Gly Asp Cys Val Arg Thr Tyr Arg

2120

2125

2130

Pro Glu Ile Lys Lys Gly Ser Tyr Asn Asn Ile Val Val His Val

2135

2140

2145

Lys Thr Ala Val Ala Asp Asn Leu Leu Phe Tyr Leu Gly Ser Ala

2150

2155

2160

Lys Phe Ile Asp Phe Leu Ala Ile Glu Met Arg Lys Gly Lys Val

2165

2170

2175

Ser Phe Leu Trp Ile Val Gly Ser Gly Val Gly Arg Val Gly Phe

2180

2185

2190

Pro Asp Leu Thr Ile Asp Asp Ser Tyr Trp Tyr Arg Ile Glu Ala

2195

2200

2205

Ser Arg Thr Gly Arg Asn Gly Ser Ile Ser Val Arg Ala Leu Asp
2210 2215 2220

Gly Pro Lys Ala Ser Met Val Pro Ser Thr Tyr His Ser Val Ser
2225 2230 2235

Pro Pro Gly Tyr Thr Ile Leu Asp Val Asp Ala Asn Ala Met Leu
2240 2245 2250

Phe Val Gly Gly Leu Thr Gly Lys Ile Lys Lys Ala Asp Ala Val
2255 2260 2265

Arg Val Ile Thr Phe Thr Gly Cys Met Gly Glu Thr Tyr Phe Asp
2270 2275 2280

Asn Lys Pro Ile Gly Leu Trp Asn Phe Arg Glu Lys Glu Gly Asp
2285 2290 2295

Cys Lys Gly Cys Thr Val Ser Pro Gln Val Glu Asp Ser Glu Gly

2300

2305

2310

Thr Ile Gln Phe Asp Gly Glu Gly Tyr Ala Leu Val Ser Arg Pro

2315

2320

2325

Ile Arg Trp Tyr Pro Asn Ile Ser Thr Val Met Phe Lys Phe Arg

2330

2335

2340

Thr Phe Ser Ser Ser Ala Leu Leu Met Tyr Leu Ala Thr Arg Asp

2345

2350

2355

Leu Lys Asp Phe Met Ser Val Glu Leu Ser Asp Gly His Val Lys

2360

2365

2370

Val Ser Tyr Asp Leu Gly Ser Gly Met Thr Ser Val Val Ser Asn

2375

2380

2385

Gln Asn His Asn Asp Gly Lys Trp Lys Ala Phe Thr Leu Ser Arg

2390

2395

2400

Ile Gln Lys Gln Ala Asn Ile Ser Ile Val Asp Ile Asp Ser Asn
2405 2410 2415

Gln Glu Glu Asn Val Ala Thr Ser Ser Ser Gly Asn Asn Phe Gly
2420 2425 2430

Leu Asp Leu Lys Ala Asp Asp Lys Ile Tyr Phe Gly Gly Leu Pro
2435 2440 2445

Thr Leu Arg Asn Leu Ser Met Lys Ala Arg Pro Glu Val Asn Val
2450 2455 2460

Lys Lys Tyr Ser Gly Cys Leu Lys Asp Ile Glu Ile Ser Arg Thr
2465 2470 2475

Pro Tyr Asn Ile Leu Ser Ser Pro Asp Tyr Val Gly Val Thr Lys
2480 2485 2490

Gly Cys Ser Leu Glu Asn Val Asn Thr Val Ser Phe Pro Lys Pro

2495

2500

2505

Gly Phe Val Glu Leu Ala Ala Val Ser Ile Asp Val Gly Thr Glu

2510

2515

2520

Ile Asn Leu Ser Phe Ser Thr Arg Asn Glu Ser Gly Ile Ile Leu

2525

2530

2535

Leu Gly Ser Gly Gly Thr Leu Thr Pro Pro Arg Arg Lys Arg Arg

2540

2545

2550

Gln Thr Thr Gln Ala Tyr Tyr Ala Ile Phe Leu Asn Lys Gly Arg

2555

2560

2565

Leu Glu Val His Leu Ser Ser Gly Thr Arg Thr Met Arg Lys Ile

2570

2575

2580

Val Ile Lys Pro Glu Pro Asn Leu Phe His Asp Gly Arg Glu His

2585

2590

2595

Ser Val His Val Glu Arg Thr Arg Gly Ile Phe Thr Val Gln Ile
2600 2605 2610

Asp Glu Asp Arg Arg His Ile Gln Asn Leu Thr Glu Glu Gln Pro
2615 2620 2625

Ile Glu Val Lys Lys Leu Phe Val Gly Gly Ala Pro Pro Glu Phe
2630 2635 2640

Gln Pro Ser Pro Leu Arg Asn Ile Pro Ala Phe Gln Gly Cys Val
2645 2650 2655

Trp Asn Leu Val Ile Asn Ser Ile Pro Met Asp Phe Ala Gln Pro
2660 2665 2670

Ile Ala Phe Lys Asn Ala Asp Ile Gly Arg Cys Thr Tyr Gln Lys
2675 2680 2685

Pro Arg Glu Asp Glu Ser Glu Ala Val Pro Ala Glu Val Ile Val

2690

2695

2700

Gln Pro Gln Ser Val Pro Thr Pro Ala Phe Pro Phe Pro Val Pro

2705

2710

2715

Thr Met Val His Gly Pro Cys Val Ala Glu Ser Glu Pro Ala Leu

2720

2725

2730

Leu Thr Gly Ser Lys Gln Phe Gly Leu Ser Arg Asn Ser His Ile

2735

2740

2745

Ala Ile Val Phe Asp Asp Thr Lys Val Lys Asn Arg Leu Thr Ile

2750

2755

2760

Glu Leu Glu Val Arg Thr Glu Ala Glu Ser Gly Leu Leu Phe Tyr

2765

2770

2775

Met Gly Arg Ile Asn His Ala Asp Phe Gly Thr Val Gln Leu Arg

2780

2785

2790

Asn Gly Phe Pro Phe Phe Ser Tyr Asp Leu Gly Ser Gly Ser Thr
2795 2800 2805

Arg Thr Met Ile Pro Thr Lys Ile Asn Asp Gly Gln Trp His Lys
2810 2815 2820

Ile Lys Ile Val Arg Val Lys Gln Glu Gly Ile Leu Tyr Val Asp
2825 2830 2835

Asp Ala Ser Ser Gln Thr Ile Ser Pro Lys Lys Ala Asp Ile Leu
2840 2845 2850

Asp Val Gly Gly Ile Leu Tyr Val Gly Gly Leu Pro Ile Asn Tyr
2855 2860 2865

Thr Thr Arg Arg Ile Gly Pro Val Thr Tyr Ser Leu Asp Gly Cys
2870 2875 2880

Val Arg Asn Leu His Met Glu Gln Ala Pro Val Asp Leu Asp Gln

2885

2890

2895

Pro Thr Ser Ser Phe His Val Gly Thr Cys Phe Ala Asn Ala Glu

2900

2905

2910

Ser Gly Thr Tyr Phe Asp Gly Thr Gly Phe Gly Lys Ala Val Gly

2915

2920

2925

Gly Phe Ile Val Gly Leu Asp Leu Leu Val Glu Phe Glu Phe Arg

2930

2935

2940

Thr Thr Arg Pro Thr Gly Val Leu Leu Gly Ile Ser Ser Gln Lys

2945

2950

2955

Met Asp Gly Met Gly Ile Glu Met Ile Asp Glu Lys Leu Met Phe

2960

2965

2970

His Val Asp Asn Gly Ala Gly Arg Phe Thr Ala Ile Tyr Asp Ala

2975

2980

2985

Glu Ile Pro Gly His Met Cys Asn Gly Gln Trp Tyr Lys Val Thr
2990 2995 3000

Ala Lys Lys Ile Lys Asn Arg Leu Glu Leu Val Val Asp Gly Asn
3005 3010 3015

Gln Val Asp Ala Gln Ser Pro Asn Ser Ala Ser Thr Ser Ala Asp
3020 3025 3030

Thr Asn Asp Pro Val Phe Val Gly Gly Phe Pro Gly Gly Leu Asn
3035 3040 3045

Gln Phe Gly Leu Thr Thr Asn Ile Arg Phe Arg Gly Cys Ile Arg
3050 3055 3060

Ser Leu Lys Leu Thr Lys Gly Thr Ala Asn Arg Trp Arg Leu Ile
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Leu Pro Arg Pro Trp Asn

3080

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Gly Glu Pro Gly Arg Gly Arg Gln Gly Gln Pro Leu Pro Trp Glu Leu

10 15 20

cgc ttg ggc cta ctt cta agt gtg ctg gct gcc aca ttg gcc cag gcc 152

Arg Leu Gly Leu Leu Leu Ser Val Leu Ala Ala Thr Leu Ala Gln Ala

25	30	35	
ccg tcc ttg gat gta cct ggc tgt tot cga gga agc tgc tat cca gcc			200
Pro Ser Leu Asp Val Pro Gly Cys Ser Arg Gly Ser Cys Tyr Pro Ala			
40	45	50	
acc ggt gac ctg ttg gtg ggc cgt gog gac aga ctg acg gcc tca tcc			248
Thr Gly Asp Leu Leu Val Gly Arg Ala Asp Arg Leu Thr Ala Ser Ser			
55	60	65	
acg tgt ggc ttg cat agc cct caa ccc tac tgt att gtc agt cac ctg			296
Thr Cys Gly Leu His Ser Pro Gln Pro Tyr Cys Ile Val Ser His Leu			
70	75	80	85
cag gac gaa aag aag tgt ttc ctg tgt gac tcc cga cgt ccc ttc tot			344
Gln Asp Glu Lys Lys Cys Phe Leu Cys Asp Ser Arg Arg Pro Phe Ser			
90	95	100	
gct cga gac aac cca aat agt cat cgg atc cag aat gta gtc acc agc			392
Ala Arg Asp Asn Pro Asn Ser His Arg Ile Gln Asn Val Val Thr Ser			
105	110	115	
ttt gcg cca caa cgc cgg acg gcc tgg tgg caa tcg gag aac ggg gtt			440
Phe Ala Pro Gln Arg Arg Thr Ala Trp Trp Gln Ser Glu Asn Gly Val			
120	125	130	

cca atg gtc acc atc caa ctg gac ctg gaa gct gag ttt cat ttc acc	488
Pro Met Val Thr Ile Gln Leu Asp Leu Glu Ala Glu Phe His Phe Thr	
135 140 145	
cac ctc att atg acg ttc aag acg ttc cgg cct got gct atg ctg gtg	536
His Leu Ile Met Thr Phe Lys Thr Phe Arg Pro Ala Ala Met Leu Val	
150 155 160 165	
gag cgt tct gca gac ttt ggc cgc acc tgg cac gtg tac cga tat ttt	584
Glu Arg Ser Ala Asp Phe Gly Arg Thr Trp His Val Tyr Arg Tyr Phe	
170 175 180	
tcc tat gac tgc ggg gct gac ttc ccg gga atc cca ctg gcc ccg cca	632
Ser Tyr Asp Cys Gly Ala Asp Phe Pro Gly Ile Pro Leu Ala Pro Pro	
185 190 195	
cgt cgc tgg gat gat gta gtg tgt gag tcc cgc tac tca gaa atc gag	680
Arg Arg Trp Asp Asp Val Val Cys Glu Ser Arg Tyr Ser Glu Ile Glu	
200 205 210	
ccg tct acg gaa ggc gag gtc atc tat cgt gtg ctg gac cct gct att	728
Pro Ser Thr Glu Gly Glu Val Ile Tyr Arg Val Leu Asp Pro Ala Ile	
215 220 225	
cct atc cca gac ccc tac agc tca cgg att cag aac ctg ttg aag atc	776
Pro Ile Pro Asp Pro Tyr Ser Ser Arg Ile Gln Asn Leu Leu Lys Ile	

230	235	240	245	
acc aac cta cga gtg aac tta acc cgg ctt cac aca ctg gga gac aac				824
Thr Asn Leu Arg Val Asn Leu Thr Arg Leu His Thr Leu Gly Asp Asn				
250	255	260		
ttg ctt gac cca cgg agg gag atc cgg gaa aaa tac tat tat gct ctc				872
Leu Leu Asp Pro Arg Arg Glu Ile Arg Glu Lys Tyr Tyr Tyr Ala Leu				
265	270	275		
tat gaa ctt gtc atc cgt ggc aac tgc ttc tgc tat ggc cac gcc tca				920
Tyr Glu Leu Val Ile Arg Gly Asn Cys Phe Cys Tyr Gly His Ala Ser				
280	285	290		
cag tgt gcg cct gca cca ggg gcg ccg gcc cat got gag ggc atg gta				968
Gln Cys Ala Pro Ala Pro Gly Ala Pro Ala His Ala Glu Gly Met Val				
295	300	305		
cac gga gcc tgt atc tgc aag cac aat act cgt gga ctc aac tgt gag				1016
His Gly Ala Cys Ile Cys Lys His Asn Thr Arg Gly Leu Asn Cys Glu				
310	315	320	325	
cag tgt cag gat ttc tat cag gac ctt ccc tgg cac cct gca gag gac				1064
Gln Cys Gln Asp Phe Tyr Gln Asp Leu Pro Trp His Pro Ala Glu Asp				
330	335	340		

ggc cat act cac gcc tgt cgg aag tgt gag tgc aac ggg cat act cat 1112

Gly His Thr His Ala Cys Arg Lys Cys Glu Cys Asn Gly His Thr His

345

350

355

agc tgc cac ttt gac atg gct gtc tac ctg goa tot gga aat gta agt 1160

Ser Cys His Phe Asp Met Ala Val Tyr Leu Ala Ser Gly Asn Val Ser

360

365

370

gga ggc gta tgc gat ggg tgt cag cac aac aca gct ggg cgc cat tgt 1208

Gly Gly Val Cys Asp Gly Cys Gln His Asn Thr Ala Gly Arg His Cys

375

380

385

gag ttc tgc cgg ccc ttc ttc tac cgt gac ccc acc aag gac atg cgg 1256

Glu Phe Cys Arg Pro Phe Phe Tyr Arg Asp Pro Thr Lys Asp Met Arg

390

395

400

405

gac cca gct gtg tgc cgt cct tgt gac tgt gac cct atg ggt tct caa 1304

Asp Pro Ala Val Cys Arg Pro Cys Asp Cys Asp Pro Met Gly Ser Gln

410

415

420

gat ggt ggt cgc tgt gat tct cat gat gac cct gtg cta gga ctg gtc 1352

Asp Gly Gly Arg Cys Asp Ser His Asp Asp Pro Val Leu Gly Leu Val

425

430

435

tca ggc cag tgt cgc tgc aaa gaa cac gtg gtt ggc act cgc tgc cag 1400

Ser Gly Gln Cys Arg Cys Lys Glu His Val Val Gly Thr Arg Cys Gln

440	445	450	
caa tgc cgt gat ggc ttc ttt gga ctt agt gcc agt gac cct cga ggg			1448
Gln Cys Arg Asp Gly Phe Phe Gly Leu Ser Ala Ser Asp Pro Arg Gly			
455	460	465	
tgc cag cgt tgc cag tgt aat tca cgg ggc aca gtg cct ggg agc tcc			1496
Cys Gln Arg Cys Gln Cys Asn Ser Arg Gly Thr Val Pro Gly Ser Ser			
470	475	480	485
cct tgt gac tcc agt agt gga acc tgt ttc tgc aag cgt ctg gtg acc			1544
Pro Cys Asp Ser Ser Ser Gly Thr Cys Phe Cys Lys Arg Leu Val Thr			
490	495	500	
gga cat ggc tgt gac cgc tgt ctg cct ggc cac tgg ggc ctg agc cat			1592
Gly His Gly Cys Asp Arg Cys Leu Pro Gly His Trp Gly Leu Ser His			
505	510	515	
gac ctg ctg ggc tgc cgt ccc tgt gac tgt gat gtg ggc ggt gcc ttg			1640
Asp Leu Leu Gly Cys Arg Pro Cys Asp Cys Asp Val Gly Gly Ala Leu			
520	525	530	
gat cct cag tgt gat gag gcc acc ggt cag tgc cgc tgc cgc caa cac			1688
Asp Pro Gln Cys Asp Glu Ala Thr Gly Gln Cys Arg Cys Arg Gln His			
535	540	545	

atg att ggg cgg cgc tgc gaa caa gtg cag cct ggc tac ttc cgg cct 1736

Met Ile Gly Arg Arg Cys Glu Gln Val Gln Pro Gly Tyr Phe Arg Pro

550 555 560 565

ttt ctg gac cat tta acc tgg gag gct gag gct gcc caa ggg cag ggg 1784

Phe Leu Asp His Leu Thr Trp Glu Ala Glu Ala Ala Gln Gly Gln Gly

570 575 580

ctt gag gtg gta gag cgg ctg gtg acc aac cga gag act ccg tcc tgg 1832

Leu Glu Val Val Glu Arg Leu Val Thr Asn Arg Glu Thr Pro Ser Trp

585 590 595

act ggc cca ggc ttt gtg cgg ctg cga gaa ggt cag gaa gtg gag ttc 1880

Thr Gly Pro Gly Phe Val Arg Leu Arg Glu Gly Gln Glu Val Glu Phe

600 605 610

ctg gtg acc tot ttg cct agg gcc atg gac tat gac ctg cta ctg cgc 1928

Leu Val Thr Ser Leu Pro Arg Ala Met Asp Tyr Asp Leu Leu Leu Arg

615 620 625

tgg gag ccc cag gtc cct gag caa tgg gca gag ctg gaa ctg atg gtg 1976

Trp Glu Pro Gln Val Pro Glu Gln Trp Ala Glu Leu Glu Leu Met Val

630 635 640 645

cag cgt ccg ggg cct gtg tot gct cac agt ccg tgc ggg cat gtg ctg 2024

Gln Arg Pro Gly Pro Val Ser Ala His Ser Pro Cys Gly His Val Leu

650	655	660	
cct aag gat gac cgc att cag ggg atg ctt cac cca aac acc agg ttt			2072
Pro Lys Asp Asp Arg Ile Gln Gly Met Leu His Pro Asn Thr Arg Phe			
665	670	675	
ttg gtg ttt ccc aga cct gtc tgc ctt gag cct ggc atc tcc tac aag			2120
Leu Val Phe Pro Arg Pro Val Cys Leu Glu Pro Gly Ile Ser Tyr Lys			
680	685	690	
ctg aag ctg aaa ctg atc gga aca ggg gga cga gcc cag cct gaa acc			2168
Leu Lys Leu Lys Leu Ile Gly Thr Gly Gly Arg Ala Gln Pro Glu Thr			
695	700	705	
tcc tac tct gga tta ctc att gac tcg ctg gtc ctg cag ccc cac gtc			2216
Ser Tyr Ser Gly Leu Leu Ile Asp Ser Leu Val Leu Gln Pro His Val			
710	715	720	725
ttg gtg cta gag atg ttt agt ggg ggc gat gct gct gct ctg gag cgc			2264
Leu Val Leu Glu Met Phe Ser Gly Gly Asp Ala Ala Ala Leu Glu Arg			
730	735	740	
cgt acc acc ttt gaa cgc tac cgc tgc cat gag gaa ggt ctg atg ccc			2312
Arg Thr Thr Phe Glu Arg Tyr Arg Cys His Glu Glu Gly Leu Met Pro			
745	750	755	

agc aag gcc cct cta tct gag acc tgt gcc ccc ctc ctc atc agc gtg 2360

Ser Lys Ala Pro Leu Ser Glu Thr Cys Ala Pro Leu Leu Ile Ser Val

760

765

770

tcc gcc ttg atc tac aat ggc gcc ttg cca tgt cag tgt gac cct caa 2408

Ser Ala Leu Ile Tyr Asn Gly Ala Leu Pro Cys Gln Cys Asp Pro Gln

775

780

785

ggc tca ctg agt tct gaa tgc agt cct cac ggt ggc cag tgc cgg tgc 2456

Gly Ser Leu Ser Ser Glu Cys Ser Pro His Gly Gly Gln Cys Arg Cys

790

795

800

805

aaa cct gga gtg gtt gga cgc cgt tgt gat gtc tgt gct act ggc tac 2504

Lys Pro Gly Val Val Gly Arg Arg Cys Asp Val Cys Ala Thr Gly Tyr

810

815

820

tat ggc ttt ggc cct gca ggc tgt caa gcc tgc cag tgt agt cct gat 2552

Tyr Gly Phe Gly Pro Ala Gly Cys Gln Ala Cys Gln Cys Ser Pro Asp

825

830

835

gga gca ctc agt gcc ctc tgt gaa ggg act agt gga cag tgc ccc tgc 2600

Gly Ala Leu Ser Ala Leu Cys Glu Gly Thr Ser Gly Gln Cys Pro Cys

840

845

850

cga cct ggt gcc ttt ggt ctt cgc tgt gac cac tgt caa cgt ggc cag 2648

Arg Pro Gly Ala Phe Gly Leu Arg Cys Asp His Cys Gln Arg Gly Gln

855	860	865	
tgg gga ttc cct aat tgc cgg ccg tgt gtc tgc aat ggg cgt gcg gat			2696
Trp Gly Phe Pro Asn Cys Arg Pro Cys Val Cys Asn Gly Arg Ala Asp			
870	875	880	885
gag tgt gat acc cac aca ggc gct tgc ctg ggc tgc cgt gat tac acg			2744
Glu Cys Asp Thr His Thr Gly Ala Cys Leu Gly Cys Arg Asp Tyr Thr			
	890	895	900
ggg ggc gag cac tgt gaa agg tgc att gct ggt ttt cat ggg gac cca			2792
Gly Gly Glu His Cys Glu Arg Cys Ile Ala Gly Phe His Gly Asp Pro			
905	910	915	
cgg ctg cca tat ggg ggc cag tgc cgg cct tgt ccc tgc cct gaa ggc			2840
Arg Leu Pro Tyr Gly Gly Gln Cys Arg Pro Cys Pro Cys Pro Glu Gly			
920	925	930	
cct ggg agc cag cga cac ttt gct act tct tgc cac cgg gat gga tat			2888
Pro Gly Ser Gln Arg His Phe Ala Thr Ser Cys His Arg Asp Gly Tyr			
935	940	945	
tcc cag caa att gtg tgc cag tgt cga gaa ggc tac aca ggg ctt cgg			2936
Ser Gln Gln Ile Val Cys Gln Cys Arg Glu Gly Tyr Thr Gly Leu Arg			
950	955	960	965

tgt gaa gct tgt gcc ccc ggg cac ttt ggg gac cca tca aag cca ggt 2984

Cys Glu Ala Cys Ala Pro Gly His Phe Gly Asp Pro Ser Lys Pro Gly

970

975

980

ggc agg tgc caa ctg tgt gag tgc agt gga aac att gat ccc atg gac 3032

Gly Arg Cys Gln Leu Cys Glu Cys Ser Gly Asn Ile Asp Pro Met Asp

985

990

995

cct gat gcc tgt gat ccc cac acg ggg caa tgc ttg cgt tgt tta 3077

Pro Asp Ala Cys Asp Pro His Thr Gly Gln Cys Leu Arg Cys Leu

1000

1005

1010

cac aac aca gag ggg ccc cac tgt ggc tat tgc aag cct ggc ttc 3122

His Asn Thr Glu Gly Pro His Cys Gly Tyr Cys Lys Pro Gly Phe

1015

1020

1025

cat ggg caa gct gcc cga cag agc tgt cac cgc tgt acc tgc aac 3167

His Gly Gln Ala Ala Arg Gln Ser Cys His Arg Cys Thr Cys Asn

1030

1035

1040

ctt ctg ggc aca gat ccc agg cgg tgc cca tct acc gac ctg tgc 3212

Leu Leu Gly Thr Asp Pro Arg Arg Cys Pro Ser Thr Asp Leu Cys

1045

1050

1055

cat tgt gac cca agc act ggg cag tgc cca tgc ctt ccc cat gtc 3257

His Cys Asp Pro Ser Thr Gly Gln Cys Pro Cys Leu Pro His Val

1060	1065	1070	
caa ggc ctc	aac tgt gac cat tgt	gcc ccc aac ttt tgg	aac ttc 3302
Gln Gly Leu	Asn Cys Asp His Cys	Ala Pro Asn Phe Trp	Asn Phe
1075	1080	1085	
acc agt ggc	cgt ggc tgc cag cct	tgt gct tgt cac cca	agc cgg 3347
Thr Ser Gly	Arg Gly Cys Gln Pro	Cys Ala Cys His Pro	Ser Arg
1090	1095	1100	
gcc aga ggc	cct acc tgc aat gag	ttc aca ggg cag tgt	cac tgt 3392
Ala Arg Gly	Pro Thr Cys Asn Glu	Phe Thr Gly Gln Cys	His Cys
1105	1110	1115	
cat gct ggc	ttt ggt ggg agg act	tgt tct gag tgc caa	gag ctc 3437
His Ala Gly	Phe Gly Gly Arg Thr	Cys Ser Glu Cys Gln	Glu Leu
1120	1125	1130	
tac tgg gga	gac cct ggt ctg cag	tgc cgt gcc tgt gac	tgt gat 3482
Tyr Trp Gly	Asp Pro Gly Leu Gln	Cys Arg Ala Cys Asp	Cys Asp
1135	1140	1145	
cct aga gga	ata gac aaa cct cag	tgt cat cgt tcc aca	ggc cac 3527
Pro Arg Gly	Ile Asp Lys Pro Gln	Cys His Arg Ser Thr	Gly His
1150	1155	1160	

tgt agc tgc	cgc cca ggc gtg tct	ggc gtg cgc tgt gac	cag tgt	3572
Cys Ser Cys	Arg Pro Gly Val Ser	Gly Val Arg Cys Asp	Gln Cys	
1165	1170	1175		
gct cgt ggc	ttc tca ggg gtt ttt	cct gct tgt cac ccc	tgc cac	3617
Ala Arg Gly	Phe Ser Gly Val Phe	Pro Ala Cys His Pro	Cys His	
1180	1185	1190		
gct tgc ttt	gga gac tgg gat cgt	gtg gta cag gac ctg	gct gct	3662
Ala Cys Phe	Gly Asp Trp Asp Arg	Val Val Gln Asp Leu	Ala Ala	
1195	1200	1205		
cgg acg cgg	cgc ctg gag cag tgg	gct cag gag ttg cag	caa aca	3707
Arg Thr Arg	Arg Leu Glu Gln Trp	Ala Gln Glu Leu Gln	Gln Thr	
1210	1215	1220		
gga gtg ctg	ggc gcc ttt gag agc	agc ttt ttg aac atg	cag ggg	3752
Gly Val Leu	Gly Ala Phe Glu Ser	Ser Phe Leu Asn Met	Gln Gly	
1225	1230	1235		
aag cta ggc	atg gtg cag gcc att	atg agt gcc cgc aat	gcc tca	3797
Lys Leu Gly	Met Val Gln Ala Ile	Met Ser Ala Arg Asn	Ala Ser	
1240	1245	1250		
gcc gcc tct	acg gcg aag ctt gta	gag gcc aca gag gga	cta cgt	3842
Ala Ala Ser	Thr Ala Lys Leu Val	Glu Ala Thr Glu Gly	Leu Arg	

1255	1260	1265	
cat gaa atc	ggg aag acc acc gag	cgc ctg act cag tta	gaa gca 3887
His Glu Ile	Gly Lys Thr Thr Glu	Arg Leu Thr Gln Leu	Glu Ala
1270	1275	1280	
gag cta aca	gct gtg cag gat gag	aac ttc aat gcc aac	cat gca 3932
Glu Leu Thr	Ala Val Gln Asp Glu	Asn Phe Asn Ala Asn	His Ala
1285	1290	1295	
ctc agt ggt	ctg gag aga gac ggg	ctt gcg ctt aat ctc	acc ctg 3977
Leu Ser Gly	Leu Glu Arg Asp Gly	Leu Ala Leu Asn Leu	Thr Leu
1300	1305	1310	
agg cag ctg	gat cag cat ctg gag	atc ctc aaa cat tca	aat ttc 4022
Arg Gln Leu	Asp Gln His Leu Glu	Ile Leu Lys His Ser	Asn Phe
1315	1320	1325	
tta ggt gcc	tat gac agc atc cga	cat gcc cac agc cag	tcc aca 4067
Leu Gly Ala	Tyr Asp Ser Ile Arg	His Ala His Ser Gln	Ser Thr
1330	1335	1340	
gag gca gag	cgc cgt gcc aac gcc	tcc acc ttt gca gta	ccc agc 4112
Glu Ala Glu	Arg Arg Ala Asn Ala	Ser Thr Phe Ala Val	Pro Ser
1345	1350	1355	

cct gtg agc	aac tca gca gat acc	cgg cgt cgg acg gaa	gtg cta	4157
Pro Val Ser	Asn Ser Ala Asp Thr	Arg Arg Arg Thr Glu	Val Leu	
1360	1365	1370		
atg ggt gcc	caa aaa gaa aac ttc	aac cgc caa cat ttg	gcc aac	4202
Met Gly Ala	Gln Lys Glu Asn Phe	Asn Arg Gln His Leu	Ala Asn	
1375	1380	1385		
cag cag gca	ctg gga cgg ctc tct	gca cat gcc cac acc	ctg agc	4247
Gln Gln Ala	Leu Gly Arg Leu Ser	Ala His Ala His Thr	Leu Ser	
1390	1395	1400		
ctg acg ggc	ata aat gag ttg gtg	tgt ggg gca cca ggg	gac gca	4292
Leu Thr Gly	Ile Asn Glu Leu Val	Cys Gly Ala Pro Gly	Asp Ala	
1405	1410	1415		
ccc tgt gcc	acc agc cct tgt ggg	ggg gcc gga tgt cgg	gat gaa	4337
Pro Cys Ala	Thr Ser Pro Cys Gly	Gly Ala Gly Cys Arg	Asp Glu	
1420	1425	1430		
gat ggg cag	ccc cgt tgt ggt ggc	ctc ggt tgc agt ggg	gca gca	4382
Asp Gly Gln	Pro Arg Cys Gly Gly	Leu Gly Cys Ser Gly	Ala Ala	
1435	1440	1445		
gcc acg gca	gat cta gcg ctg ggc	cgg gct cgg cac acg	cag gca	4427
Ala Thr Ala	Asp Leu Ala Leu Gly	Arg Ala Arg His Thr	Gln Ala	

1450	1455	1460	
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Glu Leu Gln Arg Ala Leu Val Glu Gly Gly Gly Ile Leu Ser Arg			
1465	1470	1475	
gtg tct gag act cgt cgg cag gca gaa gag gca cag cag cga gca			4517
Val Ser Glu Thr Arg Arg Gln Ala Glu Glu Ala Gln Gln Arg Ala			
1480	1485	1490	
cag gca gcc ctg gac aag gct aat gct tcc agg ggc cag gtg gaa			4562
Gln Ala Ala Leu Asp Lys Ala Asn Ala Ser Arg Gly Gln Val Glu			
1495	1500	1505	
cag gcc aat cag gag ott cga gaa ott atc cag aat gtg aaa gac			4607
Gln Ala Asn Gln Glu Leu Arg Glu Leu Ile Gln Asn Val Lys Asp			
1510	1515	1520	
ttc ctc agc cag gag gga gcc gat cct gac agt att gaa atg gta			4652
Phe Leu Ser Gln Glu Gly Ala Asp Pro Asp Ser Ile Glu Met Val			
1525	1530	1535	
gag act cgg gtg cta gac atc tcc atc cgg gcc tca ccc gag cag			4697
Ala Thr Arg Val Leu Asp Ile Ser Ile Pro Ala Ser Pro Glu Gln			
1540	1545	1550	

atc cag cgc	cta gcc agt gag att	gca gaa cgc gtc cga	agc ctg	4742
Ile Gln Arg	Leu Ala Ser Glu Ile	Ala Glu Arg Val Arg	Ser Leu	
1555	1560	1565		
gcc gac gtg	gac aca atc ctg gcc	cat acc atg ggc gac	gtg cgt	4787
Ala Asp Val	Asp Thr Ile Leu Ala	His Thr Met Gly Asp	Val Arg	
1570	1575	1580		
cgg gct gaa	cag cta ctg caa gat	gcg cac cgg gca cgg	agc cgg	4832
Arg Ala Glu	Gln Leu Leu Gln Asp	Ala His Arg Ala Arg	Ser Arg	
1585	1590	1595		
gcc gag ggt	gag aga cag aag gca	gag aca gtc caa gcg	gca ctg	4877
Ala Glu Gly	Glu Arg Gln Lys Ala	Glu Thr Val Gln Ala	Ala Leu	
1600	1605	1610		
gag gag got	cag agg gca caa gga	gct gct cag ggt gcc	atc tgg	4922
Glu Glu Ala	Gln Arg Ala Gln Gly	Ala Ala Gln Gly Ala	Ile Trp	
1615	1620	1625		
gga gca gtg	gtt gac aca caa aac	aca gag cag acc ctg	cag cgg	4967
Gly Ala Val	Val Asp Thr Gln Asn	Thr Glu Gln Thr Leu	Gln Arg	
1630	1635	1640		
gtc cag gag	agg atg gca ggt gca	gag aag tct ctg aac	tct gcc	5012
Val Gln Glu	Arg Met Ala Gly Ala	Glu Lys Ser Leu Asn	Ser Ala	

1645	1650	1655	
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Gly Glu Arg Ala Arg Gln Leu Asp Ala Leu Leu Glu Ala Leu Lys			
1660	1665	1670	
ctg aaa cgg gca gga aat agc ctg gca gca tct aca gcg gaa gaa			5102
Leu Lys Arg Ala Gly Asn Ser Leu Ala Ala Ser Thr Ala Glu Glu			
1675	1680	1685	
aca gca ggc agt gcc cag agc cgt gcc agg gag gct gag aaa caa			5147
Thr Ala Gly Ser Ala Gln Ser Arg Ala Arg Glu Ala Glu Lys Gln			
1690	1695	1700	
cta cgg gaa caa gta ggt gac caa tac caa aca gtg agg gcg ttg			5192
Leu Arg Glu Gln Val Gly Asp Gln Tyr Gln Thr Val Arg Ala Leu			
1705	1710	1715	
gca gag cgg aag gct gaa ggt gtt ctg gct gca caa gcc agg gca			5237
Ala Glu Arg Lys Ala Glu Gly Val Leu Ala Ala Gln Ala Arg Ala			
1720	1725	1730	
gaa caa ctg cgg gat gag gct cgg gac ctg ttg cag gcc gct cag			5282
Glu Gln Leu Arg Asp Glu Ala Arg Asp Leu Leu Gln Ala Ala Gln			
1735	1740	1745	

gat aag ctg cag cgg cta cag gag ctg gag ggc aca tat gag gag 5327

Asp Lys Leu Gln Arg Leu Gln Glu Leu Glu Gly Thr Tyr Glu Glu

1750

1755

1760

aac gag cgt gca ctg gag ggc aaa gcg gcc cag ctg gat ggg ctg 5372

Asn Glu Arg Ala Leu Glu Gly Lys Ala Ala Gln Leu Asp Gly Leu

1765

1770

1775

gaa gcc agg atg cgc agt gtg ctc cag gcc atc aac ttg cag gtc 5417

Glu Ala Arg Met Arg Ser Val Leu Gln Ala Ile Asn Leu Gln Val

1780

1785

1790

cag atc tac aac acc tgc cag tga ccactcccta gggcctagcc ttgtcgccaa 5471

Gln Ile Tyr Asn Thr Cys Gln

1795

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Leu Pro Trp Glu Leu Arg Leu Gly Leu Leu Leu Ser Val Leu Ala Ala
20 25 30

Thr Leu Ala Gln Ala Pro Ser Leu Asp Val Pro Gly Cys Ser Arg Gly
35 40 45

Ser Cys Tyr Pro Ala Thr Gly Asp Leu Leu Val Gly Arg Ala Asp Arg
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Ser Glu Asn Gly Val Pro Met Val Thr Ile Gln Leu Asp Leu Glu Ala

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Glu Phe His Phe Thr His Leu Ile Met Thr Phe Lys Thr Phe Arg Pro

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Ala Ala Met Leu Val Glu Arg Ser Ala Asp Phe Gly Arg Thr Trp His

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Val Tyr Arg Tyr Phe Ser Tyr Asp Cys Gly Ala Asp Phe Pro Gly Ile

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Pro Leu Ala Pro Pro Arg Arg Trp Asp Asp Val Val Cys Glu Ser Arg

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Tyr Ser Glu Ile Glu Pro Ser Thr Glu Gly Glu Val Ile Tyr Arg Val

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Leu Asp Pro Ala Ile Pro Ile Pro Asp Pro Tyr Ser Ser Arg Ile Gln

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Asn Leu Leu Lys Ile Thr Asn Leu Arg Val Asn Leu Thr Arg Leu His

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Tyr Gly His Ala Ser Gln Cys Ala Pro Ala Pro Gly Ala Pro Ala His

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Ala Glu Gly Met Val His Gly Ala Cys Ile Cys Lys His Asn Thr Arg

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Gly Leu Asn Cys Glu Gln Cys Gln Asp Phe Tyr Gln Asp Leu Pro Trp

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His Pro Ala Glu Asp Gly His Thr His Ala Cys Arg Lys Cys Glu Cys

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Asn Gly His Thr His Ser Cys His Phe Asp Met Ala Val Tyr Leu Ala

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Ala Gly Arg His Cys Glu Phe Cys Arg Pro Phe Phe Tyr Arg Asp Pro

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Pro Met Gly Ser Gln Asp Gly Gly Arg Cys Asp Ser His Asp Asp Pro

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Val Leu Gly Leu Val Ser Gly Gln Cys Arg Cys Lys Glu His Val Val

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Gly Thr Arg Cys Gln Gln Cys Arg Asp Gly Phe Phe Gly Leu Ser Ala

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Ser Asp Pro Arg Gly Cys Gln Arg Cys Gln Cys Asn Ser Arg Gly Thr

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Val Pro Gly Ser Ser Pro Cys Asp Ser Ser Ser Gly Thr Cys Phe Cys

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Lys Arg Leu Val Thr Gly His Gly Cys Asp Arg Cys Leu Pro Gly His

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Trp Gly Leu Ser His Asp Leu Leu Gly Cys Arg Pro Cys Asp Cys Asp

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Val Gly Gly Ala Leu Asp Pro Gln Cys Asp Glu Ala Thr Gly Gln Cys

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Arg Cys Arg Gln His Met Ile Gly Arg Arg Cys Glu Gln Val Gln Pro

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Gly Tyr Phe Arg Pro Phe Leu Asp His Leu Thr Trp Glu Ala Glu Ala

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Ala Gln Gly Gln Gly Leu Glu Val Val Glu Arg Leu Val Thr Asn Arg

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Glu Thr Pro Ser Trp Thr Gly Pro Gly Phe Val Arg Leu Arg Glu Gly

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Ala Gln Pro Glu Thr Ser Tyr Ser Gly Leu Leu Ile Asp Ser Leu Val
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Leu Gln Pro His Val Leu Val Leu Glu Met Phe Ser Gly Gly Asp Ala

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Ala Ala Leu Glu Arg Arg Thr Thr Phe Glu Arg Tyr Arg Cys His Glu

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Glu Gly Leu Met Pro Ser Lys Ala Pro Leu Ser Glu Thr Cys Ala Pro

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Leu Leu Ile Ser Val Ser Ala Leu Ile Tyr Asn Gly Ala Leu Pro Cys

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775

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Gln Cys Asp Pro Gln Gly Ser Leu Ser Ser Glu Cys Ser Pro His Gly

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790

795

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Cys Arg Asp Tyr Thr Gly Gly Glu His Cys Glu Arg Cys Ile Ala Gly
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Pro Cys Pro Glu Gly Pro Gly Ser Gln Arg His Phe Ala Thr Ser Cys

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Tyr Thr Gly Leu Arg Cys Glu Ala Cys Ala Pro Gly His Phe Gly Asp

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Pro Ser Lys Pro Gly Gly Arg Cys Gln Leu Cys Glu Cys Ser Gly Asn

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Ile Asp Pro Met Asp Pro Asp Ala Cys Asp Pro His Thr Gly Gln Cys

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1005

Leu Arg Cys Leu His Asn Thr Glu Gly Pro His Cys Gly Tyr Cys

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Ser Thr Gly His Cys Ser Cys Arg Pro Gly Val Ser Gly Val Arg

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Cys Asp Gln Cys Ala Arg Gly Phe Ser Gly Val Phe Pro Ala Cys

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His Pro Cys His Ala Cys Phe Gly Asp Trp Asp Arg Val Val Gln

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Asp Leu Ala Ala Arg Thr Arg Arg Leu Glu Gln Trp Ala Gln Glu

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His Ser Asn Phe Leu Gly Ala Tyr Asp Ser Ile Arg His Ala His

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Thr Glu Val Leu Met Gly Ala Gln Lys Glu Asn Phe Asn Arg Gln

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His Leu Ala Asn Gln Gln Ala Leu Gly Arg Leu Ser Ala His Ala

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Val Arg Ser Leu Ala Asp Val Asp Thr Ile Leu Ala His Thr Met

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Gly Asp Val Arg Arg Ala Glu Gln Leu Leu Gln Asp Ala His Arg

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1590

Ala Arg Ser Arg Ala Glu Gly Glu Arg Gln Lys Ala Glu Thr Val

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1605

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Glu Ala Leu Lys Leu Lys Arg Ala Gly Asn Ser Leu Ala Ala Ser

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1695

Ala Glu Lys Gln Leu Arg Glu Gln Val Gly Asp Gln Tyr Gln Thr

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Thr Tyr Glu Glu Asn Glu Arg Ala Leu Glu Gly Lys Ala Ala Gln

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Ser Cys Tyr Asp Gly Val Gly Arg Ala Gln Arg Cys Leu Pro Glu Phe

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Glu Asn Ala Ala Phe Gly Arg Arg Ala Glu Ala Ser His Thr Cys Gly

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Arg Pro Pro Glu Asp Phe Cys Pro His Val Gly Ala Pro Gly Ala Gly

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Leu Gln Cys Gln Arg Cys Asp Asp Ala Asp Pro Gly Arg Arg His Asp
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Lys Phe His Thr Ser Arg Pro Glu Ser Phe Ala Ile Tyr Lys Arg Thr
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Pro Trp Ala Arg Gly Thr Ala Glu Asp Ala Asn Glu Cys Leu Pro Cys
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Asn Cys Ser Gly His Ser Glu Glu Cys Thr Phe Asp Arg Glu Leu Tyr
340 345 350

Arg Ser Thr Gly His Gly Gly His Cys Gln Arg Cys Arg Asp His Thr
355 360 365

Thr Gly Pro His Cys Glu Arg Cys Glu Lys Asn Tyr Tyr Arg Trp Ser
370 375 380

Pro Lys Thr Pro Cys Gln Pro Cys Asp Cys His Pro Ala Gly Ser Leu

385 390 395 400

Ser Leu Gln Cys Asp Asn Ser Gly Val Cys Pro Cys Lys Pro Thr Val

405 410 415

Thr Gly Trp Lys Cys Asp Arg Cys Leu Pro Gly Phe His Ser Leu Ser

420 425 430

Glu Gly Gly Cys Arg Pro Cys Ala Cys Asn Val Ala Gly Ser Leu Gly

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Thr Cys Asp Pro Arg Ser Gly Asn Cys Pro Cys Lys Glu Asn Val Glu

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Gly Gly Ser Arg Gly Phe Asn Cys Glu Ser Lys Pro Glu Pro Glu Glu

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Thr Cys Phe Asp Lys Tyr Thr Gly Asn Thr Tyr Arg Val Gly Asp Thr

65 70 75 80

Tyr Glu Arg Pro Lys Asp Ser Met Ile Trp Asp Cys Thr Cys Ile Gly

85 90 95

Ala Gly Arg Gly Arg Ile Ser Cys Thr Ile Ala Asn Arg Cys His Glu

100 105 110

Gly Gly Gln Ser Tyr Lys Ile Gly Asp Thr Trp Arg Arg Pro His Glu

115 120 125

Thr Gly Gly Tyr Met Leu Glu Cys Val Cys Leu Gly Asn Gly Lys Gly

130 135 140

Glu Trp Thr Cys Lys Pro Ile Ala Glu Lys Cys Phe Asp Gln Ala Ala

145 150 155 160

Gly Thr Ser Tyr Val Val Gly Glu Thr Trp Glu Lys Pro Tyr Gln Gly

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Tyr Arg Ile Gly Asp Thr Trp Ser Lys Lys Asp Asn Arg Gly Asn Leu
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Leu Gln Cys Ile Cys Thr Gly Asn Gly Arg Gly Glu Trp Lys Cys Glu
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Arg His Thr Ser Leu Gln Thr Thr Ser Ala Gly Ser Gly Ser Phe Thr
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Asp Val Arg Thr Ala Ile Tyr Gln Pro Gln Pro His Pro Gln Pro Pro
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Pro Tyr Gly His Cys Val Thr Asp Ser Gly Val Val Tyr Ser Val Gly

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Met Gln Trp Leu Lys Thr Gln Gly Asn Lys Gln Met Leu Cys Thr Cys

290

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300

Leu Gly Asn Gly Val Ser Cys Gln Glu Thr Ala Val Thr Gln Thr Tyr

305

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315

320

Gly Gly Asn Ser Asn Gly Glu Pro Cys Val Leu Pro Phe Thr Tyr Asn

325

330

335

Gly Lys Thr Phe Tyr Ser Cys Thr Thr Glu Gly Arg Gln Asp Gly His

340

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350

Leu Trp Cys Ser Thr Thr Ser Asn Tyr Glu Gln Asp Gln Lys Tyr Ser

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Phe Cys Thr Asp His Thr Val Leu Val Gln Thr Arg Gly Gly Asn Ser

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Thr Cys Phe Gly Gln Gly Arg Gly Arg Trp Lys Cys Asp Pro Val Asp

515

520

525

Gln Cys Gln Asp Ser Glu Thr Arg Thr Phe Tyr Gln Ile Gly Asp Ser

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535

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Trp Glu Lys Tyr Leu Gln Gly Val Arg Tyr Gln Cys Tyr Cys Tyr Gly

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Arg Gly Ile Gly Glu Trp Ala Cys Gln Pro Leu Gln Thr Tyr Pro Asp

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Asn Ser His Pro Ile Gln Trp Ser Ala Pro Glu Ser Ser His Ile Ser
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Gly Gln Arg Glu Val Thr Arg Phe Asp Phe Thr Thr Thr Ser Thr Ser
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675 680 685

Pro Val Val Ala Thr Ser Glu Ser Val Thr Glu Ile Thr Ala Ser Ser
690 695 700

Phe Val Val Ser Trp Val Ser Ala Ser Asp Thr Val Ser Gly Phe Arg
705 710 715 720

Val Glu Tyr Glu Leu Ser Glu Glu Gly Asp Glu Pro Gln Tyr Leu Asp
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Leu Pro Ser Thr Ala Thr Ser Val Asn Ile Pro Asp Leu Leu Pro Gly
740 745 750

Arg Lys Tyr Thr Val Asn Val Tyr Glu Ile Ser Glu Glu Gly Glu Gln
755 760 765

Asn Leu Ile Leu Ser Thr Ser Gln Thr Thr Ala Pro Asp Ala Pro Pro
770 775 780

Asp Pro Thr Val Asp Gln Val Asp Asp Thr Ser Ile Val Val Arg Trp

785 790 795 800

Ser Arg Pro Arg Ala Pro Ile Thr Gly Tyr Arg Ile Val Tyr Ser Pro

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Ser Val Glu Gly Ser Ser Thr Glu Leu Asn Leu Pro Glu Thr Ala Asn

820 825 830

Ser Val Thr Leu Ser Asp Leu Gln Pro Gly Val Gln Tyr Asn Ile Thr

835 840 845

Ile Tyr Ala Val Glu Glu Asn Gln Glu Ser Thr Pro Val Phe Ile Gln

850 855 860

Gln Glu Thr Thr Gly Val Pro Arg Ser Asp Lys Val Pro Pro Pro Arg

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Ile Val Gly Tyr Arg Leu Thr Val Gly Leu Thr Arg Gly Gly Gln Pro

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Asn Leu Gln Pro Gly Ser Glu Tyr Ala Val Ser Leu Val Ala Val

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Lys Gly Asn Gln Gln Ser Pro Arg Val Thr Gly Val Phe Thr Thr

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Leu Gln Pro Leu Gly Ser Ile Pro His Tyr Asn Thr Glu Val Thr

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Glu Thr Thr Ile Val Ile Thr Trp Thr Pro Ala Pro Arg Ile Gly

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Pro Ala Val Pro Pro Pro Thr Asp Leu Arg Phe Thr Asn Val Gly

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Thr Pro Thr Ser Leu Leu Ile Ser Trp Asp Ala Pro Ala Val Thr

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Val Arg Tyr Tyr Arg Ile Thr Tyr Gly Glu Thr Gly Gly Ser Ser

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Pro Val Gln Glu Phe Thr Val Pro Gly Ser Lys Ser Thr Ala Thr

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Val Gln Thr Ala Val Thr Thr Ile Pro Ala Pro Thr Asn Leu Lys

1595

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1605

Phe Thr Gln Val Thr Pro Thr Ser Leu Thr Ala Gln Trp Thr Ala

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Pro Asn Val Gln Leu Thr Gly Tyr Arg Val Arg Val Thr Pro Lys

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Glu Lys Thr Gly Pro Met Lys Glu Ile Asn Leu Ala Pro Asp Ser

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Ser Ser Val Val Val Ser Gly Leu Met Val Ala Thr Lys Tyr Glu

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Val Ser Val Tyr Ala Leu Lys Asp Thr Leu Thr Ser Arg Pro Ala

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Val Arg Ser Tyr Thr Ile Thr Gly Leu Gln Pro Gly Thr Asp Tyr
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Lys Ile His Leu Tyr Thr Leu Asn Asp Asn Ala Arg Ser Ser Pro
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Val Val Ile Asp Ala Ser Thr Ala Ile Asp Ala Pro Ser Asn Leu

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Pro Pro Arg Ala Arg Ile Thr Gly Tyr Ile Ile Lys Tyr Glu Lys

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Pro Gly Ser Pro Pro Arg Glu Val Val Pro Arg Pro Arg Pro Gly

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Val Thr Glu Ala Thr Ile Thr Gly Leu Glu Pro Gly Thr Glu Tyr

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1840

1845

Thr Ile Gln Val Ile Ala Leu Lys Asn Asn Gln Lys Ser Glu Pro

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1855

1860

Leu Ile Gly Arg Lys Lys Thr Asp Glu Leu Pro Gln Leu Val Thr

1865

1870

1875

Leu Pro His Pro Asn Leu His Gly Pro Glu Ile Leu Asp Val Pro
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Ser Thr Val Gln Lys Thr Pro Phe Ile Thr Asn Pro Gly Tyr Asp
1895 1900 1905

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Ser Leu Gly Gln Gln Met Ile Phe Glu Glu His Gly Phe Arg Arg
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Thr Thr Pro Pro Thr Thr Ala Thr Pro Val Arg His Arg Pro Arg
1940 1945 1950

Pro Tyr Pro Pro Asn Val Asn Glu Glu Ile Gln Ile Gly His Val
1955 1960 1965

Pro Arg Gly Asp Val Asp His His Leu Tyr Pro His Val Val Gly

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1975

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Leu Asn Pro Asn Ala Ser Thr Gly Gln Glu Ala Leu Ser Gln Thr

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1990

1995

Thr Ile Ser Trp Thr Pro Phe Gln Glu Ser Ser Glu Tyr Ile Ile

2000

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Ser Gly His Phe Arg Cys Asp Ser Ser Lys Trp Cys His Asp Asn
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Gly Val Asn Tyr Lys Ile Gly Glu Lys Trp Asp Arg Gln Gly Glu
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Asn Gly Gln Met Met Ser Cys Thr Cys Leu Gly Asn Gly Lys Gly
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Glu Phe Lys Cys Asp Pro His Glu Ala Thr Cys Tyr Asp Asp Gly

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Lys Thr Tyr His Val Gly Glu Gln Trp Gln Lys Glu Tyr Leu Gly

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